ISSN 0355-1180

UNIVERSITY OF HELSINKI

Department of Food and Environmental Sciences

EKT Series 1826

UTILIZATION OF BOVINE GLYCOSYLATED CASEINOMACROPEPTIDE (CMP) BY BIFIDOBACTERIA AND ITS GROWTH-PROMOTING EFFECT

Wan Lih Ching

Helsinki 2018

ABSTRACT

HELSINGIN YLIOPISTO – HELSINGFORS UNIVERSITET – UNIVERSITY OF HELSINKI

Tiedekunta/Osasto – Fakultet/Sektion – Faculty		Laitos/Institution– Department
Faculty of Agriculture and Forestry		Department of Food and Environmental Sciences
Tekijä/Författare – Author		
Wan Lih Ching		
Työn nimi / Arbetets titel – Title		
Utilization of bovine glycosylated caseinomacropeptide (CMP) by bifidobacteria and its growth-promoting effect		
Oppiaine /Läroämne – Subject		
Food Science (Bioprocessing)		
Työn laji/Arbetets art – Level	Aika/Datum – Month and year	Sivumäärä/ Sidoantal – Number of pages
M. Sc. thesis	February 2018	80

Tiivistelmä/Referat – Abstract

Bifidobacteria confer various health benefits to the host. Bifidobacterial growth can be promoted by caseinomacropeptide (CMP), which is the potential oligosaccharides-bearing portion of the κ-casein (κ-CN) in milk. This project was carried out to study the utilization of bovine glycosylated CMP by *Bifidobacterium infantis* ATCC 15697, *B. bifidum* ATCC 29521, and *B. longum* ATCC 15707 as well as to investigate the growth-promoting effect of CMP on these strains.

Two types of media, namely the basal chemically-defined medium (bCDM) and the nutrient-rich medium known as de Man-Rogosa-Sharpe (MRS), were employed to achieve respective research objectives. The formula of the bCDM was developed by addition technique. It was found that L-cysteine, magnesium and manganese were needed by all the strains, and additionally, thiamin and nicotinic acid were essential for the growth of *B. bifidum* ATCC 29521. Since more than 70% of the glycosylated chains attached to a CMP are terminated with sialic acid residues, the cell counts and bound sialic acid contents in the media after incubation were analysed. Higher cell counts and lower bound sialic acid contents were found in the inoculated media supplemented with CMP as compared to unsupplemented group. Unexpectedly, slower microbial death, instead of growth, was observed in all inoculated bCDM+CMP in comparison to bCDM. It was also surprising to discover that *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 can utilize the glycosylated chains despite that no sialidase has ever been isolated from them.

As a conclusion, *B. infantis* ATCC 15697 can cleave and utilize sialic acids, but not other glycans, that are attached to the glycosylated CMPs; while both *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 can cleave terminal sialic acid residues, followed by the utilization of the glycans attached to the glycosylated CMPs other than sialic acids. It was also found that bovine CMP can exert a similar growth-promoting effect on all three strains. From an industrial point of view, it is feasible to add the bovine CMP into the bifidobacterial growth medium to enhance their growth. Further study is warranted to characterize the sialidases present in *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707.

Avainsanat – Nyckelord – Keywords

Caseinomacropeptide, *O*-linked glycosylation, bifidobacteria, sialidase, chemically-defined medium, acidic ninhydrin, growth-promoting.

Säilytyspaikka – Förvaringställe – Where deposited

The Digital Repository of University of Helsinki, HELDA

Muita tietoja – Övriga uppgifter – Additional information

EKT Series 1826

PREFACE

This master's thesis project was initiated and supervised by Ir. Hein van Valenberg from the Dairy Science and Technology Department of Wageningen University in the Netherlands. The laboratory work took place from February 2017 to June 2017 in the Food Quality and Design Group.

I am profoundly grateful to Prof. Dr. Eddy Smid from the Food Microbiology Department of the same university for his guidance and suggestions. A special thanks to Arla Foods Amba (Denmark) for supplying Lacprodan CGMP-20. This project would have been greatly delayed without it.

I wish to express my gratitude to the lab assistants Ing. Ingrid Maas, Erik Meulenbroeks, Frans Lettink, and Xandra Bakker-de Haan in Wageningen University. Apart from that, I must thank all members of Food Quality and Design Group for helping me settle in and maintaining a motivational environment.

Last but not least, I particularly appreciate the permission granted by Prof. Kati Katina and the International Exchange Services at the University of Helsinki for me to carry out my master's thesis project during an Erasmus exchange. I would also like to thank Dr. Pekka Varmanen to be the advisor of my thesis.

Thank you!

Wan Lih CHING

Helsinki, 2018

TABLE OF CONTENTS

List of Figures

List of Tables

List of Abbreviations

ABC = ATP-binding cassette
ACE = angiotensin-I-converting enzyme
ANOVA = one-way analysis of variance
ATCC = American Type Culture Collection
B. bifidum = *Bifidobacterium bifidum*
B. infantis = *Bifidobacterium longum* subsp. *infantis*
B. longum = *Bifidobacterium longum* subsp. *longum*
bCDM = basal chemically-defined medium
CMP = caseinomacropeptide
DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany)
endoBI-1 = endo-β-*N*-acetylglucosaminidase
Gal = galactose
Gal*N*Ac = *N*-acetylgalactosamine
Gal*N*AcT = UDP-Gal*N*Ac:polypeptide *N*-acetylgalactosaminyl transferase
GLBP = galacto-*N*-biose/lacto-*N*-biose I-binding protein
GNB = galacto-*N*-biose
HMOs = human milk oligosaccharides
HPLC = high performance liquid chromatography
IgE = immunoglobulin E
JCM = Japan Collection of Microorganisms
LMG = Laboratorium voor Microbiologie, Universiteit Gent (Belgium)
MFGM = milk fat globule membrane
MRS = de Man-Rogosa-Sharpe
Neu*N*Ac = *N*-acetylneuraminic acid; or sialic acid
NK cell = natural killer cell
OD = optical density
PBS = phosphate-buffered saline
PFZ = physiological salt solution
PNA = peanut lectin agglutinin
spp. = species
subsp. = subspecies
TBA = thiobarbituric acid
TCA = trichloroacetic acid
UV-Vis = ultraviolet-visible (spectrophotometer)
v/v = volume per volume
w/v = weight per volume
w/w = weight per weight
κ-CN = κ-casein

Abbreviations for amino acids

Ala = alanine
Asn = asparagine
Asp = aspartic acid
Cys = cysteine
Gln = glutamine
Glu = glutamic acid
Gly = glycine
Ile = isoleucine
Leu = leucine
Lys = lysine
Met = methionine
Phe = phenylalanine
Pro = proline
Ser = serine
Thr = threonine
Val = valine

1 INTRODUCTION

1.1 Problem definition

Human gut microbiota is composed of approximately 160 species per individual, and *Bifidobacterium* spp. is one of them (Rodríguez *et al.*, 2015). Bifidobacteria confer benefits to the host including, but not limited to, the prevention of intestinal infection, the synthesis of pyridoxine (vitamin B_6), and the enhancement of gut immunity (Shah, 2002). Therefore, supporting their growth is desirable, both from the consumer point of view and the industrial point of view (for instance, functional ingredients manufacturers).

One of the substances that benefit these commensal microorganisms is oligosaccharides. Oligosaccharides are found not only in fruits and vegetables but also in milk. Human milk oligosaccharides (HMOs) have been studied extensively in the recent decades in relation to their prebiotic effects (Bode, 2009; Coppa *et al.*, 2004). However, human milk is not readily available in normal households. Instead, bovine milk is vastly produced all around the world including European countries (Eurostat, 2017). Thus, it is much more readily available than human milk.

Bovine milk contains oligosaccharides in free- and bound-form. One example of the latter is glycoproteins. They can present in the casein fraction, whey fraction, and milk fat globule membrane (MFGM) fraction of the milk (O'Riordan *et al.*, 2014). Among the casein family (α-, β-, κ-, γ-casein), only κ-casein (κ-CN) can conjugate with carbohydrate. K-CN is composed of 169 amino acids, and the potential carbohydrate-bearing portion is 64-amino acid from the carbon terminal. It is known as caseinomacropeptide (CMP) and can be found in the rennet whey during cheese-making. Approximately 40% of the rennet whey generated in the European dairy industry is used as livestock feed, as fertilizer, or simply discharged as waste (Macwan *et al.*, 2016).

CMP is worth an attention as it has been demonstrated to promote bifidobacterial growth (Azuma *et al.*, 1984; Cicvárek *et al.*, 2010). This growth-promoting activity was claimed to be attributed by both the polypeptide as well as the associated glycans. However, contradictory results were also found and the researchers argued that the growth-promoting effect was only because of the polypeptide itself (Poch &

Bezkorovainy, 1991; Robitaille, 2013). Since the data available to date is non-conclusive, this project was aimed to gain insight of the potential beneficial effect of bovine CMP on the growth of bifidobacteria, emphasizing on the bifidobacterial ability to utilize the glycosylated chains.

1.2 Research objectives

To achieve the goal, the objectives of this project were, firstly, to study the utilization of bovine glycosylated CMP by *Bifidobacterium longum* subsp. *infantis* (*B. infantis*) ATCC 15697, *Bifidobacterium bifidum* (*B. bifidum*) ATCC 29521, and *Bifidobacterium longum* subsp. *longum* (*B. longum*) ATCC 15707; and secondly, to investigate the growth-promoting effect of CMP on these strains.

Following that, two research questions were formulated. First, is glycosylated CMP utilizable by *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521, and *B. longum* ATCC 15707? And second, does CMP confer a growth-promoting effect on these strains?

1.3 Hypotheses

B. infantis, *B. bifidum*, and *B. longum* were selected since they are commonly added into dairy products such as yogurt and cheese (Lourens-Hattingh & Viljoen, 2001). Nevertheless, they differ from one another in terms of the availability of carbohydrate-metabolizing enzymes, among all aspects. One major difference among these three strains is the production of sialidases. *B. infantis* possesses sialidase, *B. bifidum* produces a similar battery of enzymes like *B. infantis* except that it only liberates sialic acid residues but leaves them unutilized, whereas *B. longum* does not produce sialidases (Ward *et al.*, 2007).

Hence, it was hypothesized that meanwhile *B. infantis* can fully utilize the glycosylated CMP as the building blocks for its growth, *B. bifidum* can utilize all glycans attached to the glycosylated CMP except sialic acids; whereas *B. longum* can only utilize a minimal amount of the attached carbohydrate, since sialic acid is the terminal residue

of more than 70% of the glycan chains attached to a glycosylated CMP. CMP represents a source of carbon and nitrogen, thus its supplementation into the media was expected to enhance the growth (with the effect intensity in descending order) of *B. infantis*, *B. bifidum*, and *B. longum*.

2 LITERATURE REVIEW

2.1 *Bifidobacterium* spp.

The first strain of *Bifidobacterium* spp. was isolated from infant feces at the beginning of the 20th century by Tissier and it was named as *Bacillus bifidus* (Shah, 2002). The microbe had also been assigned to other genera such as *Bacteroides*, *Lactobacillus* and *Corynebacterium* over the years. It was not until 1960's when bifidobacteria and lactobacilli were distinguished from one another based on, for instance, the presence of enzymes: fructose-6-phosphate-phosphoketolase was considered specific for bifidobacteria, whereas aldolase and glucose-6-phosphate dehydrogenase were considered specific for lactobacilli (Shah, 2002). The *Bifidobacterium* genus covers 32 species to date, five of which are substantially used in the dairy industry, namely *B. adolescentis*, *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* (Shah, 2002).

Bifidobacteria account up to 91% of the microbiota in newborns while representing 3-7% in adults (Martinez *et al.*, 2013). They are Gram-positive and non-spore forming bacteria. Most of them are catalase-negative and intolerant to oxygen. Bifidobacteria are mesophilic microbes where those of human origin proliferate most rapidly at 36-38°C. They grow optimally at pH 6.5-7.0 but hardly grow below pH 5.0 or above 8.0. The growth of *Bifidobacterium* spp. produces acetic acid and lactic acid, often in the ratio of 3:2 (Shah, 2002).

The technological aptitudes of the strains play an important role in determining their application in dairy products. For example, *B. infantis* ATCC 15697 and *B. longum*

ATCC 15707 are resistant to bile salt (Ibrahim & Bezkorovainy, 1993). This indicates that the strains can survive the exposure to bile salt in the small intestine and then populate in the colon. Although these strains barely survive in gastric pH (of around 2), but their survival rate increases when the pH is around 4 (Vernazza *et al.*, 2005), which often occurs after the ingestion of a meal. Growth-promoting factors can also be added to the delivery medium (such as yogurt or cheese) to enhance their viability, and CMP is potentially one of them. Further elaboration can be found in Section 2.2.

2.1.1 Health benefits of oral bifidobacteria administration

Oral administration of bifidobacteria, for example, through the consumption of bifidobacteria-containing dairy products, can improve health in several ways as explained below. It should, however, be noted that the benefits are strain-specific.

2.1.1.1 Production of antimicrobial compounds

Bifidobacteria can help in controlling the population of pathogens in the gastrointestinal tract through the production of antimicrobial compounds. Bifidobacteria produce organic acids, particularly lactic acid, which lower the pH of the environment thereby inhibiting pathogens from localized colonization. Henriksson & Conway (2001) had also demonstrated that the strains Lafti^{TM} B74 and Lafti^{TM} B97 can inhibit the growth of *Salmonella typhimurium*.

Bacteriocins are antimicrobial peptides that act against other bacteria but do not have an effect towards the producing organisms themselves. Few bifidobacterial isolates from infant stool, composing of *B. longum, B. bifidum* and *B. adolescentis*, have been shown to produce heat-stable proteinaceous compounds that were antagonistic against five to nine strains of *Listeria monocytogenes* (Touré *et al.*, 2003). *Bifidobacterium animalis* subsp. *lactis* (*B. lactis*) Bb-12 and *B. longum* Bb-46 can produce bifilact Bb-12 and bifilong Bb-46, respectively, which were highly inhibitory against *S. typhimurium*, *Staphylococcus aureus*, *Bacillus aureus* and *Escherichia coli* (Saleh & El-Sayed, 2005). Bifidin I from *B. infantis* BCRC 14602 (same as ATCC 15697) is another example of

Bifidobacterium-associated bacteriocins. It was active against a wide range of Gram-positive and Gram-negative bacteria, including *L. monocytogenes* LSD 346, *S. aureus* AS 1.72, *B. cereus* ATCC 14574, *E. coli* TG1, and *S. typhimurium* ATCC 29631 (Cheikhyoussef *et al.*, 2010; Touré *et al.*, 2003).

2.1.1.2 Stimulation of immune function

The dietary intake of fermented dairy foods containing bifidobacteria can modulate cellular function related to both natural immunity and acquired immunity. Consumption of *B. lactis* HN019 or *B. lactis* Bb-12 can enhance the phagocytic activity of leucocytes and macrophages *in vivo* (Gill *et al.*, 2000; Schiffrin *et al.*, 1997). Phagocytic activity of these immune cells plays an important role in removing pathogens and cell debris. Furthermore, antigen-specific antibody responses can also be stimulated after the ingestion of *B. lactis* HN019, leading to a rapid recovery from bacterial or viral infections (Gill *et al.*, 2000; Shu *et al.*, 2001).

Research also showed that *B. lactis* HN019 can improve the activity of circulating natural killer (NK) cells, contributing to a better tumor surveillance in the body, among all advantages (Gill *et al.*, 2001). An *in vitro* study has reported that *B. bifidum* LMG 13195 can induce the secretion of chemokines and interferon molecules that recruit lymphocytes to fight against human colon cancer cells (López *et al.*, 2012). Hence, dietary supplementation of bifidobacteria can result in an increased resistance to tumorigenesis, especially against colorectal tumors. Besides that, oral bifidobacteria administration can reduce the incidence and severity of inflammatory diseases via regulating the production of pro-inflammatory cytokines. Studies have concluded that *B. longum* NCC 2705 and *B. breve* Yakult strain have such an ability to regulate the functioning of T cells, consequently preventing intestinal inflammation or even ameliorating chronic inflammatory bowel diseases such as ulcerative colitis (Jeon *et al.*, 2012; Zhang *et al.*, 2015). The oral intake of *B. longum* BB536 and *B. lactis* Bb-12 is also suggested to prevent allergic diseases. This is because they can inhibit immunoglobulin E (IgE) production, thereby lowering the allergen-induced immune responses such as hay fever (Feleszko *et al.*, 2007; Takahashi *et al.*, 2006).

Apart from that, high daily intake of bifidobacteria such as *B. lactis* HN019 and *B. longum* BB536 for a period of time can positively modulate the colonic microbiota, relieving the severity of gastrointestinal disorders (Miglioranza *et al.*, 2015). An example of such health problem is lactose intolerance. Lactose intolerance is related to the incomplete digestion of lactose and inefficient colonic processing of the lactose metabolites. Despite that bifidobacteria cannot improve the lactose digestion in the small intestine, they flourish in the colon following a regular lactose ingestion (He *et al.*, 2007). Thus, bifidobacteria are present at a higher number in the colon, in addition to an adaptation to lactose consumption (Hertzler & Savaiano, 1996). This indicates that more lactose can be hydrolyzed by the bacteria and the colon can absorb the fermentation metabolites more efficiently, and consequently alleviating the gastrointestinal symptoms such as diarrhea (He *et al.*, 2006).

2.1.1.3 Improvement in lipid profile

The ingestion of bifidobacteria can improve the serum lipid profile of the consumer. For instance, the total serum cholesterol and low-density lipoprotein cholesterol of patients with metabolic syndrome or hypercholesterolemia had significant decrease after a daily intake of *B. lactis* HN019 or *B. longum* BL1 for a period of time (Bernini *et al.*, 2016; Xiao *et al.*, 2003). It was proposed that the binding of cholesterol by the bacteria during cell proliferation lowers the absorption of cholesterol in the intestine. Another mechanism could be that the bifidobacteria produce hydrolase that deconjugate bile acid (Kim *et al.*, 2004), resulting in a higher excretion of the free bile acid. Following this, the liver synthesizes new bile acid by drawing more cholesterol from the bloodstream. Furthermore, the fermentation of *B. longum* B1536 in soymilk can yield bioactive peptides with high inhibitory activity on angiotensin-I-converting enzyme (ACE) (Donkor *et al.*, 2007). This means that the ingestion of the soymilk fermented with the strain can aid in regulating blood pressure, alongside with other health benefits.

Apart from lowering the level of harmful lipid in the serum, bifidobacteria can also produce beneficial lipid molecules. *B. breve* NCFB 2258 and *B. lactis* Bb-12 are the most efficient generator of conjugated linoleic acid among the common bifidobacteria strains (Coakley *et al.*, 2003). They convert the free linoleic acid from the growth environment

into conjugated linoleic acid, which inhibits atherogenicity and reduces body fat (West *et al.*, 1998).

2.1.1.4 Synthesis of B vitamins

Bifidobacteria commonly synthesize B vitamins as metabolites. *B. infantis* ATCC 15697 and *B. adolescentis* MB239 are the example of strains carrying an ability to synthesize folate (Pompei *et al.*, 2007). The presence of folate-producing strains in the colon not only benefits the growth of other commensal bacteria, but also confers protection against colonic inflammation (Pompei *et al.*, 2007; Said, 2011).

Thiamin, nicotinic acid, pyridoxine, biotin and riboflavin can be synthesized by certain bifidobacteria strains. For example, *B. adolescentis* SI-30 synthesizes thiamin and nicotinic acid (Deguchi *et al.*, 1985), and the biosynthesis pathways of thiamin and nicotinic acid have been completely identified in *B. longum* NCC2705 (Schell *et al.*, 2002). Most strains of bifidobacteria synthesize pyridoxine at a considerable concentration (Deguchi *et al.*, 1985), whereas *B. infantis* I-10-5, *B. bifidum* A234-4, and *B. longum* M101-2 can produce different amounts of biotin depending on the types of oligosaccharides present in the media (Noda *et al.*, 1994). Riboflavin is another B vitamin that can be synthesized at varied amount by bifidobacteria. Riboflavin content increased when soymilk was fermented with *B. infantis* CCRC 14633 or *B. longum* B6 (Hou *et al.*, 2000). However, this is inconsistent with the finding from Deguchi *et al.* (1985) where excess riboflavin was not detected in the media singly-inoculated by strains of *B. infantis* and *B. longum*. The discrepancy is most probably due to the different composition of media, the strains as well as the methodology employed.

2.1.1.5 Enhancement of nutrients bioavailability

Studies have demonstrated that bifidobacteria can increase the level of amino acids, for instance leucine, during fermentation (Hou *et al.*, 2000). This is resulted from the proteolytic activities of the organisms. Consequently, the food fermented by bifidobacteria has a better protein digestibility because more protein can be absorbed and utilized.

Additionally, bifidobacteria possess β-glucosidase that hydrolyses soybean isoflavone glycosides into the corresponding aglycones which are better absorbed in human (Chen *et al.*, 2011). Flavonols (such as kaempferol) and hydroxycinnamates (such as chlorogenic acid) that predominantly present in plant-based foods can also be metabolised into bioactive compounds by many strains of bifidobacteria (Marotti *et al.*, 2007; Raimondi *et al.*, 2015).

The ability of bifidobacteria to transform inorganic minerals into organic compounds (thus more bioavailable) has also been exhibited. As example, selenium is essential in proper functioning of enzymes. *B. animalis* 01 can convert inorganic selenium into an organic form known as selenomethionine, which is more absorbable by the human gut (Zhang *et al.*, 2009). A daily intake of 50-60 μg selenium is recommended by the Nordic Council of Ministers, and the main dietary sources are meat and cereal products (Nordic Council of Ministers, 2012).

2.1.1.6 Removal of hazardous compounds

Bifidobacteria can reduce toxins and carcinogens in their growth environment. For instance, *B. bifidum* PTCC 1644 has shown to lower the aflatoxin B1, B2, G1 and G2 produced by the toxigenic *Aspergillus* spp. (Ghazvini *et al.*, 2016). This is attributed to the bifidobacterial cell wall which can effectively bind to the toxic molecules. Besides that, *B. longum* BB536 can exert antitumor protection by enhancing the activity of glutathione transferase, which efficiently transforms the carcinogens in the colon into less harmful conjugates (Challa *et al.*, 1997).

Apart from that, bifidobacteria possess antioxidative activity too. Wang *et al.* (2006) had demonstrated that the soymilk fermented by *B. infantis* ATCC 27920 or *B. longum* B6 exerted higher reducing activity than unfermented soymilk. The reason behind this phenomenon is due to the antioxidative properties of the metabolites of isoflavones and the catabolism of proteins. Furthermore, the co-fermentation of *L. acidophilus* with either one of the abovementioned strains could enhance the reducing activity (thus, antioxidative activity) of the fermented soymilk even further.

2.1.2 Growth requirements of bifidobacteria

Generally, the growth requirements of bifidobacteria are fewer than the genus *Lactobacillus*. Bifidobacteria, in fact, are capable of the biosynthesis of some amino acids, vitamins, purines and pyrimidines (Gomes & Malcata, 1999; Sela *et al.*, 2008). Many amino acids can be derived from carbohydrate intermediates. For example, glycine can potentially be synthesized from glycerate-3-phosphate, and tryptophan can potentially be synthesized from phosphoenolpyruvate (Lee & O'Sullivan, 2010). Nevertheless, cysteine is an indispensable amino acid for most bifidobacteria (Ferrario *et al.*, 2015).

Although bifidobacteria are not nutritionally fastidious, they do not grow well in milk because the concentration of small peptides and amino acids in the milk (approximately 0.1 g/L) is insufficient for their growth (Gomes & Malcata, 1999). Thus, proteolytic starter cultures such as *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* are often used to initiate the fermentation in milk, and *Bifidobacterium* spp. is then incorporated at a later stage.

The ability to ferment carbohydrate is species- and even strain-specific. Table 1 displays the carbohydrates fermentability of *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707. Generally, hexoses (such as glucose), oligosaccharides (such as fructooligosaccharides), and polysaccharides (such as amylose) are fermentable by all *Bifidobacterium* spp.

Table 1. Carbohydrate fermentation profile of *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 [modified from Gueimonde *et al.*, 2004].

Strains	Types of carbohydrate				
	Fucose	**Ribose**	**Sucrose**	**Lactose**	**Arabinose**
B. infantis ATCC 15697	+	+	-	+	-
B. bifidum ATCC 29521	-	+	+	+	-
B. longum ATCC 15707	-	-	-	+	+

+ : fermentable.
- : non-fermentable.

A minimal medium for bifidobacteria has been developed by Hassinen *et al.* (1951). By starting with a complete chemically-defined medium, the removal of thiamin, riboflavin, nicotinic acid, pyridoxine, folic acid, *p*-aminobenzoic acid, adenine, guanine, uracil and xanthine, alone or in combination, from the medium did not decrease the bifidobacterial growth. Whereas the removal of a salt solution containing $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, NaCl, and $MnSO_4.H_2O$ reduced the bifidobacterial growth, and individual elimination of any of the minerals had no effect on growth. The researchers consequently summarized that the tested bifidobacterial strains isolated from the stools of breast-fed infants would grow on a medium comprised of a fermentable carbohydrate (lactose), buffers (sodium acetate and dipotassium phosphate), minerals (in the form of the abovementioned salt solution), ammonium salts (ammonium acetate), cysteine as well as B vitamins (biotin and calcium pantothenate).

As demonstrated in several studies, exogenous B vitamins are required for normal growth of bifidobacteria. While biotin and calcium pantothenate have been shown indispensable for growth of Birch strain, Lockhart strain, Timberlain strain and Perrish strain (Hassinen *et al.*, 1951), thiamin and nicotinic acid are essential for maximal growth in majority of the strains of *B. adolescentis* (Deguchi *et al.*, 1985). Riboflavin can also be dispensable or indispensable for bifidobacteria. *B. infantis* ATCC 15697 can synthesis riboflavin, so the nutrient acts only as a growth stimulant for this strain (Sela *et al.*, 2008), but it may be required for the growth of *B. adolescentis* (Deguchi *et al.*, 1985).

Hence, the differences of growth requirements among *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 possibly lay on biotin, calcium pantothenate, thiamin, nicotinic acid, and riboflavin.

2.1.3 *B. infantis* ATCC 15697

This type strain has the synonym names of S12, DSM 20088, and JCM1222 (NCBI, n.d.). Its guanine-cytosine content is 60.5 mol% (Mattarelli *et al.*, 2008).

This strain is only present in human infant, typically in the intestines (Matsuki *et al.*, 1999). It is genetically adapted to metabolize the nutrients in human milk (Ward *et al.*, 2007). It utilizes sialylated and fucosylated oligosaccharides in the human milk through the cooperation of a wide array of permeases, extracellular carbohydrate-binding proteins, and glycoside hydrolases (Desjardins & Roy, 1990; Shah, 2002). One of the glycoside hydrolases is exo-α-sialidase, which is the enzyme that removes terminal sialic acid residues from a glycan chain (Kiyohara *et al.*, 2011).

2.1.4 *B. bifidum* ATCC 29521

This type strain has the synonym names of Tissier 1900, JCM1255, DSM 20456, and LMG 11041 (NCBI, n.d.). The guanine-cytosine content is 61 mol% (Whitman *et al.*, 2012).

The variant b of *B. bifidum* ATCC 29521, which was used in this project, predominates in the feces of human infants (Ventura *et al.*, 2003; Whitman *et al.*, 2012). Although no sialidase has ever been isolated from this strain, it exhibits sialic acid-degrading activity where sialic acids can be cleaved from sialylated glycan chains but are left unutilized (Milani *et al.*, 2015; Ward *et al.*, 2007). Additionally, the strain also produces endo-α-*N*-acetylgalactosaminidase, which is the enzyme that removes Gal*N*Ac-containing disaccharides from the polypeptide backbone in a glycoprotein (Duranti *et al.*, 2015; Fujita *et al.*, 2005).

2.1.5 *B. longum* ATCC 15707

This type strain has the synonym names of E194b (variant a), DSM 20219, JCM1217, and LMG 13197 (NCBI, n.d.). The guanine-cytosine content is 61 mol% and has a large variety of plasmids (Mattarelli *et al.*, 2008).

It predominantly colonizes in the colon and can be isolated from the feces of human infant or adult (Ventura *et al.*, 2003), though the type strain used in this project originated from adult. The strain possesses endo-α-*N*-acetylgalactosaminidase but does not have exo-α-sialidase (Fujita *et al.*, 2005; Kiyohara *et al.*, 2011).

2.2 Growth-promoting effect of caseinomacropeptide (CMP) on bifidobacteria

2.2.1 CMP as carbon and nitrogen sources

Apart from the co-inoculation with proteolytic starter cultures, it is a usual practice to add growth-promoting factors, such as whey protein permeate, into the milk during fermentation to improve the growth of *Bifidobacterium* spp. (Shah, 2002). This is not only due to the extra source of nitrogen in the form of small peptides and amino acids, but also due to the associated glycans including sialic acid (Modler, 1993).

However, enzymes availability of bifidobacteria vary from strain to strain. For example, *B. infantis* ATCC 15697 grew rapidly in the medium containing *N*-linked glycoproteins from bovine colostrum whey, but did not grow in deglycosylated milk protein fraction; whereas *B. lactis* UCD316 did not grow in either medium (Karav *et al.*, 2016). This implies that *B. infantis* can synthesize the enzyme known as endo-β-*N*-acetylglucosaminidase (endoBI-1) which enables the cleavage of *N*-linked glycan chains, thereby using them as substrates for growth; but the enzyme is absent in *B. lactis*.

Bovine CMP has exhibited growth-promoting effect on *B. infantis* S12 (same as ATCC 15697) in the study conducted by Azuma *et al.* (1984). The finding was inferred from the higher level of titratable acidity in CMP-supplemented group than in unsupplemented group after 24 hours of incubation. They proposed that both the

polypeptide moiety (which refers to the CMP backbone) as well as the attached glycans play the role in enhancing the bifidobacterial growth. Nonetheless, the growth-promoting effect of bovine CMP was about four times weaker than human CMP, probably because of the lower carbohydrate content in the former (Azuma *et al.*, 1984; Yamauchi *et al.*, 1981).

A similar finding was obtained when the rennet whey fraction of the bovine milk supported an extended growth of *B. bifidum* ATCC 11863, *B. bifidum* ATCC 15696, *B. breve* ATCC 15700, *B. infantis* ATCC 15697, and *B. longum* ATCC 15708 in the study carried out by Petschow & Talbott (1990). It was deduced from the significantly higher total acidity after 48 hours of incubation in the rennet whey-supplemented group as compared to the control group. Besides that, bovine CMP also enhances the growth of *B. lactis* DSM 10140. This was evidenced by the higher viable counts and lower pH after 6- and 24-hour incubation in the medium supplemented with bovine CMP in comparison to the unsupplemented group (Janer *et al.*, 2004).

2.2.2 CMP as nitrogen source

Nevertheless, the effectiveness of the carbohydrate moiety of the CMP to enhance bifidobacterial growth is not definitively established. Robitaille (2013) noticed that the growth rate of *B. thermophilum* RBL67 was similar in the medium supplemented either with bovine highly-glycosylated CMP, or non-glycosylated CMP, or CMP (a mixture of both). The researcher therefore concluded that CMP contributes to the bifidobacterial growth merely due to the polypeptide itself.

Poch & Bezkorovainy (1991) reported a similar phenomenon. They investigated the growth-promoting activity of trypsin-digested bovine milk where the turbidity of the media was compared after 24 hours of incubation. The researchers found out that the medium supplemented with the para-κ-CN enhanced the respective growth of *B. bifidum* ATCC 15696 and *B. longum* ATCC 15708 at a larger intensity than the effect exerted by supplementing the medium with the CMP. Therefore, they denied the importance of the carbohydrate portion in CMP. They instead pointed that the presence of disulfide/sulfhydryl residues as well as a yet-to-be-identified factor in the para-κ-CN that play the growth-promoting role. It is noteworthy that the CMP used in their study was

prepared by using trypsin, an enzyme that hydrolyses κ-CN at Lys_{111}, Lys_{112} or Lys_{116} (Bouhallab *et al.*, 1993), thus yielding different peptides than the CMP prepared by chymosin.

Apart from that, sialic acids in the glycosylated CMP were claimed not to be the reason behind its growth-stimulation on bifidobacteria. The inoculation of *B. infantis* ATCC 15697 and *B. bifidum* ATCC 29521, respectively, into a broth supplemented with 0.01 mg/mL of glycosylated CMP (containing approximately 0.6 µg sialic acid/mL) had significantly higher growth rates than the supplementation of glycosylated CMP at 1.0 mg/mL (Idota *et al.*, 1994). Another study reported a similar finding where the sialic acid content of glycosylated CMP did not have a direct relationship with growth-promoting effect on *B. lactis* Bb-12 and *B. longum* BBMN68 (Tang *et al.*, 2013).

2.3 Source of bovine CMP

About 3.5% (w/w) of bovine milk is protein (Heck *et al.*, 2009). Casein accounts for approximately three quarters of the total protein whereas whey protein constitutes the remaining proportion. There are four major types of casein, namely α-, β-, κ-, and γ-casein. K-casein (κ-CN) takes up about 8% of the casein family.

K-CN is composed of 169 amino acids. Upon the addition of rennet (an enzyme preparation) into the milk during cheese-making, chymosin in the rennet cleaves the bond between Phe_{105} and Met_{106} of the κ-CN, yielding two peptides – CMP, which is released to the liquid (known as "rennet whey" subsequently in this project), and para-κ-CN, which is hydrophobic and remains in the curd (Delfour *et al.*, 1965). The former is a polypeptide composed of 64 amino acids from the carbon terminal of κ-CN, which is starting from Met_{106} until Val_{169}. Among the 20 common amino acids, six of them are absent in CMP, namely histidine, arginine, cysteine, and the aromatic amino acids – phenylalanine, tyrosine, and tryptophan. The two most common allelic variants of κ-CN are A and B. They differ from one another at the amino acid positions 136 and 148 (Figure 1).

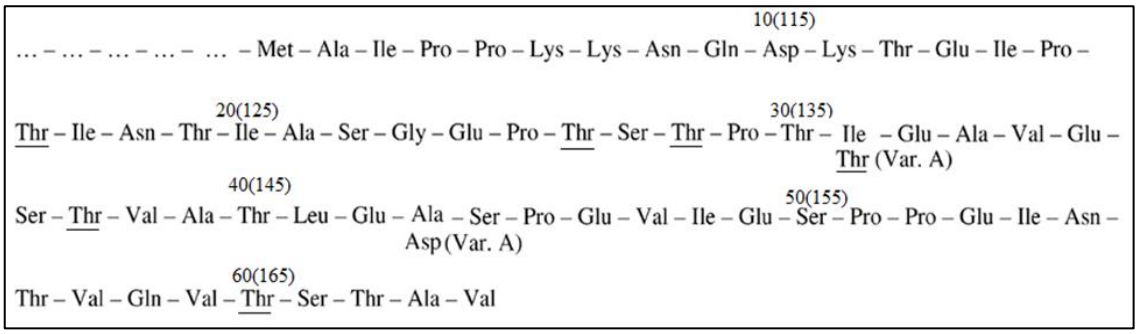


Figure 1. Structure of CMP variant B with the potential glycosylation sites underlined [modified from Thomä-Worringer et al., 2006]. The positions of the amino acid residues in the κ-CN are given in parentheses. Amino acid substitutions in CMP variant A are given in brackets.

2.4 Glycosylation of bovine CMP

Almost all proteins synthesized in eukaryotic cells undergo post-translational modifications to properly perform their molecular functions (Blom *et al.*, 2004). Glycosylation is one of the common modifications.

The building blocks of the glycan chains in glycosylated CMP are galactose (Gal), *N*-acetylgalactosamine (Gal*N*Ac), and *N*-acetylneuraminic acid (Neu*N*Ac) (Manso & López-Fandiño, 2004). Neu*N*Ac is more commonly known as "sialic acid", therefore the latter term will be used subsequently in this project. The glycosylation of a CMP is initiated by attaching a Gal*N*Ac residue to the hydroxyl group (OH^-) of a Ser or Thr residue via an *O*-linkage, which is catalyzed by the enzyme UDP-Gal*N*Ac:polypeptide *N*-acetylgalactosaminyl transferase (Gal*N*AcT) (Holland *et al.*, 2005). On the other hand, the attachment of a carbohydrate residue to the amino group (NH_2) of an Asn residue is known as *N*-linked glycosylation. It is, however, only found in other fractions of the bovine mature milk, for instance lactoferrin in the whey.

Six glycosylation sites have been proposed, of which all are Thr residues (Figure 1) (Pisano *et al.*, 1994). This finding disagreed with an earlier study conducted by Zevaco & Ribadeau-Dumas (1984) which showed that the CMP glycosylation can also occur at Ser_{141}. The number of glycan chains attached to one CMP backbone can vary from one to five (Abd El-Salam *et al.*, 1996; Dziuba & Minkiewicz, 1996). The glycosylation of a CMP was initially suggested to occur by a random mechanism (Fiat *et al.*, 1988). However, newer research has found out that the process occurs with consistent patterns (Coolbear *et al.*, 1996). The primary glycosylation site occurs at Thr_{131} (Holland *et al.*,

2005), but Thr_{133} as the primary glycosylation site has also been proposed (Hua *et al.*, 2012). Despite that, either Thr_{131} or Thr_{133} must be the primary site because of the technical difficulty in distinguishing between them (Hernandez-Hernandez *et al.*, 2010).

The glycan chains attach to a CMP molecule can be in five arrangements (Figure 2). The most abundant type of chain is tetrasaccharides (56.0%), followed by trisaccharides (18.5% for branched-chain; and 18.4% for straight-chain), disaccharides (6.3%), and lastly monosaccharide (0.8%) (Saito & Itoh, 1992). The occurrence of two isomers of the trisaccharides can be explained by the presence of two types of sialyltransferases in the mammary epithelial cells. One or two sialic acid residues are present at the terminals of the trisaccharides or tetrasaccharides chain as depicted in Figure 2. In other word, more than 70% of the glycan chains attached to a CMP contain sialic acid (Dank, 2016).

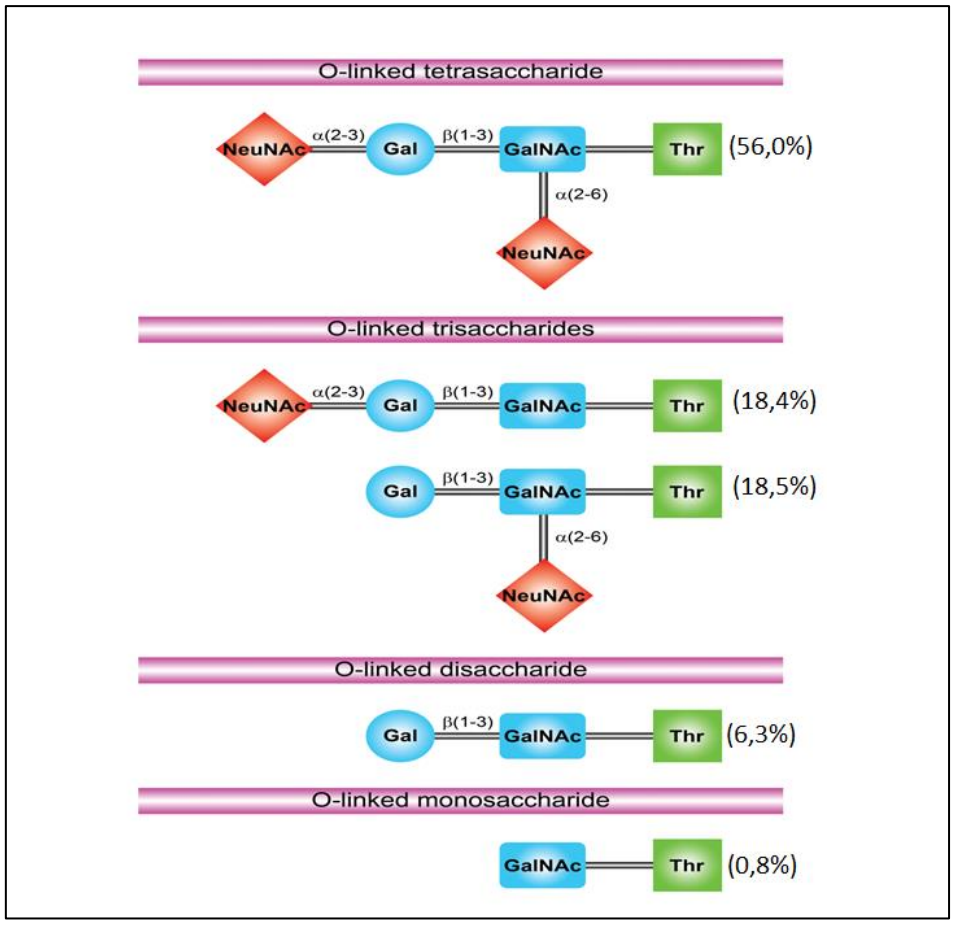


Figure 2. Percentage distribution of glycan chains attached to a CMP [modified from Bijl, 2014].

CMP variant B is generally more glycosylated than variant A (Coolbear *et al.*, 1996). The postulated reason for this phenomenon is that the presence of Thr_{136}, instead of Ile_{136}, in variant A modifies the conformation of the molecule, thereby reducing the efficiency of glycosylation process by Gal*N*AcT in the mammary epithelial cells (Robitaille *et al.*, 1991). The degree of glycosylation, as reflected by the sialic acid content in κ-CN, is highest in colostrum, decreases a little in the first 2-3 months and then increases again with progressing lactation (Robitaille *et al.*, 1991). Nonetheless, three-fifth of CMPs is glycosylated in average (O'Riordan *et al.*, 2014).

2.5 Isolation of bovine CMP

CMP is very polar due to its amino acid composition as well as the attached glycan chains (Yvon *et al.*, 1994). Thus, it is highly soluble in aqueous solutions. The isoelectric point of CMP is around pH 4.0, but can be as low as pH 3.15 when the CMP contains higher number of sialic acid residues (Kreuβ *et al.*, 2009). Based on these characteristics, several methods have been developed to isolate CMP. A combination of methods is required to enhance the purity of the product.

2.5.1 Selective precipitations

Milk samples are generally treated with chymosin to release CMPs into the rennet whey. Following that, the selective precipitation is done by adding trichloroacetic acid (TCA) into the rennet whey to precipitate proteins other than CMP, subsequently dialysis and lyophilization of the supernatant. However, one disadvantage of this approach is that a lower degree of glycosylation results in lower solubility in TCA. For instance, the final concentration of 120 g/L TCA precipitates non-glycosylated CMPs and therefore leading to reduced recovery rate (Vreeman *et al.*, 1986).

Another approach is through the alcohol precipitation after a heat treatment (Saito *et al.*, 1991). Rennet whey, which has been adjusted to pH 6.0, is first heated at 98°C for one hour before added with cold 50% (v/v) ethanol. The heat treatment coagulates whey

proteins and the addition of ethanol precipitates them. Likewise, it is followed by lyophilization of the supernatant.

2.5.2 Ultrafiltration techniques

Another means of isolating CMP is through ultrafiltration. CMP (variant A) has the molecular mass of 6.7 kDa (Jollès *et al.*, 1972), whereas highly glycosylated ones is approximately 9.6 kDa (Mollé & Léonil, 2005).

The preparation of CMP at an industrial scale often employs ultrafiltration technique because it offers the advantage of obtaining a product with higher quality and purity. The best example is the CMP product commercialised by Arla Foods Amba (Denmark) which is protected by a patent. The process is unique because a large yield of pure product can be obtained at low operating costs without noticeable denaturation (Holst & Chatterton, 2002). The main processing steps include acid precipitation preceding to the ultrafiltration at 12°C using a membrane that has a cut-off value of 20 kDa.

It is interesting to note that the apparent molecular weight of the CMP can change owing to pH-induced aggregation during the enzymatic cleavage of the κ-CN. Mikkelsen *et al.* (2005) had demonstrated that the chymosin or pepsin hydrolysis of κ-CN performed at pH 6.6 resulted in aggregated CMP units with an apparent molecular weight of 35 kDa. Contrarily, the enzymatic hydrolysis of the κ-CN carried out at pH 2.5 or 3.4 resulted in both aggregated and monomeric forms of CMP, which can have the apparent molecular weights of 9, 18, and 35 kDa. The probable reason is that at pH 6.6, κ-CN molecules tend to bind to adjacent molecules more tightly, for instance, by crosslinking via disulphide bonds at Cys_{11} and Cys_{88}. This in turn increases the association of CMP units. Whereas in an acidic environment, κ-CN molecules are less associated with one another. Despite that, the apparent molecular weight of CMP is stable against pH changes once it has been formed.

2.5.3 Chromatographic techniques

Ion-exchange chromatography is by far the most popular technique used in purifying CMPs, whereas gel chromatography and hydrophobic interaction chromatography are the alternative choices (Nakano & Ozimek, 2000; Silva-Hernandez *et al.*, 2002).

CMP remains negatively charged in an acidic environment while other whey proteins are positively charged (Kreuβ *et al.*, 2009). Thus, after adjusting the rennet whey to pH 4 and subjecting to an ion-exchanger resin, main whey proteins will adsorb on the resin and separate from the filtrate fraction which contains CMPs (Kawasaki *et al.*, 1994). The filtrate fraction can then be collected and concentrated. The separation of glycosylated from non-glycosylated fractions of CMP is also possible by using anion-exchange membrane adsorption chromatography (Robitaille, 2013). A variation succeeding the ion exchange is the use of a peanut lectin agglutinin (PNA) column to fractionate glycosylated CMP into sialylated and non-sialylated portions (Saito *et al.*, 1991).

2.6 Quantification of glycosylated CMP

The quantification of glycosylated CMP can be determined chromatographically or colorimetrically. While the general principle of the chromatography has been described in Section 2.5.3, the presence of sialic acids in glycosylated CMP forms the basis of the colorimetric methods. Sialic acid, an acylated nine-carbon molecule (Figure 3), is the condensation product of one pyruvic acid residue and one *N*-acetyl-D-mannosamine residue (Warren & Felsenfeld, 1962). Sialic acid reacts with specific reagents to form a chromophore (colored compound) and the color intensity is directly proportional to its concentration. Table 2 displays the colorimetric assays that are commonly used to quantify sialic acid.

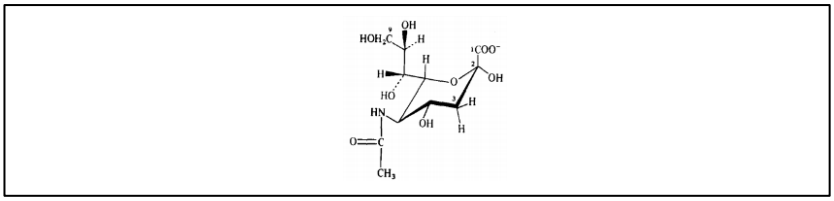


Figure 3. Structure of a sialic acid [adapted from O'Kennedy, 1988].

Table 2. Colorimetric assays commonly used in sialic acid quantification.

Assays	Pros	Cons	References
Thiobarbituric acid (TBA) Main reagents: Sodium periodate, phosphoric acid, 2-thiobarbituric acid, cyclohexanone Absorbance: 549 nm	• Reproducible results • Sensitive • Measure free sialic acid contents	• Interference by nucleic acids • Laborious	Schauer, 2012
Orcinol Main reagents: Solution with ferric ions, hydrochloric acid, orcinol, isoamyl alcohol Absorbance: 570 nm	• Rapid test	• Interference by glucose and fructose • Only measure total sialic acid contents	Schauer, 2012
Resorcinol Main reagents: Solution with cupric ions, resorcinol Absorbance: 588 nm	• Rapid test • More sensitive than orcinol assay	• Less sensitive than TBA assay • Only measure total sialic acid contents	Gottschalk, 1960
Direct Ehrlich Main reagents: *p*-dimethylaminobenzaldehyde, hydrochloric acid Absorbance: 565 nm	• Color (chromophore) is stable	• Less sensitive than TBA assay • Only measure total sialic acid contents	Gottschalk, 1960
Enzymatic Main reagents: A mixture of sialidase, sialic acid aldolase, and few other enzymes Absorbance: • 340 nm (if oxidize the pyruvate by using lactate dehydrogenase) • 630 nm (if oxidize the pyruvate by using pyruvate oxidase)	• Specific	• Costly	Dasgupta, 2015; Muniandy *et al.*, 2005
Acidic ninhydrin Main reagents: Ninhydrin, glacial acetic acid, hydrochloric acid, ethanol Absorbance: 470 nm	• Rapid test • Specific • Simple test	• Interference by cysteine, cystine and tryptophan	Yao & Ubuka, 1987

2.6.1 High-performance liquid chromatography (HPLC)

The most common chromatographic instrument used for the quantification of glycosylated CMP is by using the high-performance liquid chromatography (HPLC). The European Commission has laid down a HPLC method for the determination of glycosylated CMP as part of the effort to detect the addition of rennet whey in skimmed milk powder (European Commission, 2008).

A reversed-phase HPLC is required to perform the analysis. The eluents often consist of acetonitrile, isopropanol, and trifluoroacetic acid. Principally, the method involves selective precipitation by TCA to remove fat and proteins from the specimen prior to the injection into the HPLC. The molecular mass of the isolated peptides can further be identified by coupling the HPLC with a mass spectrometer. Nevertheless, chromatographic method is generally more sophisticated and laborious than the colorimetric methods, particularly acidic ninhydrin method.

2.6.2 Acidic ninhydrin method

Acidic ninhydrin method has served as a routine screening method for detecting milk adulteration in Brazil since 2003 (Fukuda *et al.*, 2004). Although this method is commonly used for quantifying amino acids such as tryptophan, cysteine and tyrosine (Figure 4 displays the absorption spectra of the related chromophores), but it can also be used to quantify sialic acids where the results can be as reliable as those obtained from the HPLC method published by the European Commission (Fukuda *et al.*, 2004; Yao & Ubuka, 1987).

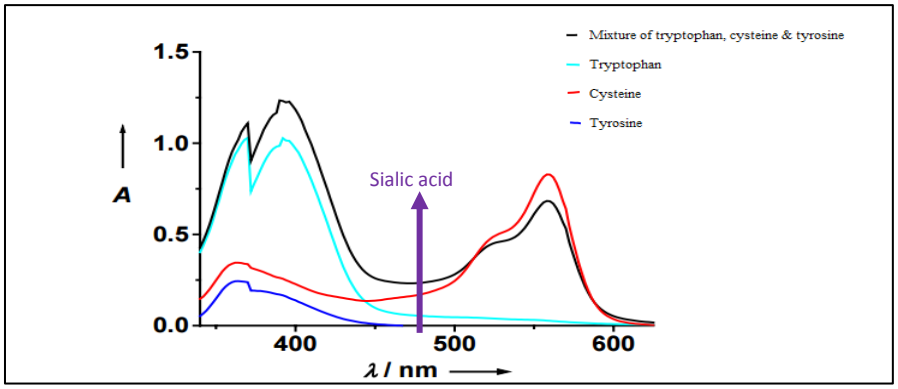


Figure 4. Absorption spectra of the chromophores formed between tryptophan, cysteine and tyrosine with the ninhydrin reagent [modified from Bao et al., 2006]. The maximum absorption of the sialic acid-formed chromophore, 470 nm, is marked in purple.

Acidic ninhydrin method involves the use of TCA to first selectively solubilize the glycoproteins that carry sialic acid residues, followed by the precipitation using phosphotungstic acid and ethanol (Inoue *et al.*, 1998; Patt & Grimer, 1974). Finally, the reaction between the acidic ninhydrin reagent with the sialic acids in the precipitate yields chromophores which have the absorption maximum at 470 nm. A duration of two hours is sufficient to complete the whole procedure, which is less time-consuming as compared to the HPLC method.

3 MATERIALS AND METHODS

3.1 Source of the bifidobacterial cultures

Freeze-dried *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521, and *B. longum* ATCC 15707 were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Germany. All three strains were grown overnight in sterilized de Man-Rogosa-Sharpe (MRS) broth (Oxoid, UK; prepared as stated on the label) supplemented with sterile-filtered 0.5 g/L L-cysteine.HCl monohydrate (Sigma, Japan) in 15mL-Greiner tubes. Then, 0.7 mL of the respective overnight culture was mixed with 0.3 mL of the 99% glycerol (Sigma, Germany) (thus, final glycerol concentration of

30%) in 1.5mL-cryo vials. All cryo vials were immediately stored in a -46°C freezer and served as the stock cultures.

Strains were activated by growing three successive times in sterilized MRS broth supplemented with sterile-filtered 0.5 g/L L-cysteine.HCl monohydrate in 15mL-Greiner tubes. The transfer volume from the stock cultures was 0.2% (v/v). Following that, 1% (v/v) was transferred to a new MRS broth each time. Incubation for *B. longum* was done at 37°C for 16 hours, and 24 hours for *B. infantis* and *B. bifidum*.

3.2 Preparation of the inocula

1 mL of each overnight culture was centrifuged (5430R, Eppendorf, Germany) in a pre-sterilized 1.5mL-Eppendorf tube at 10000 rpm for 5 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended in an equal volume of phosphate-buffered saline (PBS; the composition was 2.71 g/L $NaH_2PO_4.2H_2O$, 8.98 g/L $Na_2HPO_4.2H_2O$, 8.5 g/L NaCl, and 1 L demineralized water which was adjusted to pH 7.2 before autoclave). This step is also known as "washing". The cell pellet was washed twice before the third PBS+cell suspension was diluted with PBS to OD_{600nm} 0.10-0.35. The OD was measured using a UV-Vis spectrophotometer via its fiber optic dip probe (Varian Cary 50 Bio, Agilent, USA). The suspension, which was equivalent to approximately 10^7 cfu/mL at this stage, was then used as the inoculum for the preparation of bCDM and the first part of the growth experiments.

Meanwhile, the cell count was determined by spread-plating on a MRS agar plate [MRS + 15 g/L bacteriological agar (Oxoid, UK) + 0.5 g/L sterile-filtered L-cysteine.HCl monohydrate]. Serial dilution was performed using physiological salt solution (PFZ; Tritium Microbiologie, the Netherlands). Incubation of the agar plates was done at 37°C in an air-tight jar filled with anaerobic gas mixture (85% N_2, 10% CO_2, 5% H_2) using Anoxomat WS9000 (Mart Microbiology, the Netherlands). Additional plates of each strain were incubated aerobically at 37°C to check for possible microbial contamination.

3.3 Preparation of a basal chemically-defined medium (bCDM)

A bCDM, which was devoid of carbon and nitrogen sources, was employed to investigate the utilization of bovine glycosylated CMP by the strains. Preliminary experiments were conducted to decide the most suitable formula.

3.3.1 Preliminary experiments

A very basic formula of bCDM (Table 3) and a list of potentially indispensable nutrients were created by referring to the literature. Carbon (predetermined as D-glucose) and nitrogen sources were temporarily added to ensure that the bCDM can support the growth of the three strains. The base bCDM was sterilized by autoclave (except L-cysteine.HCl monohydrate which was sterilized by filtration) and served as the control.

Table 3. Initial preparation of the bCDM.

Nutrients/Chemicals	Concentrations (g/L)
D-glucose.H_2O	15.0
NaCl	5.0
$K_2HPO_4.3H_2O$	2.0
L-cysteine.HCl.H_2O	0.5
$MgSO_4$	0.2
$MnSO_4.H_2O$	0.1
Bromocresol purple	0.03
(Demineralized water)	(1 L)

Eight nitrogen sources were tested for suitability by adding into the base bCDM at single addition. After single-strain inoculation with the suspension prepared in Section 3.2 followed by an overnight incubation, the optimum nitrogen source was selected and proceeded to the subsequent step.

In the subsequent experiment, six B vitamins and four nucleobases were added into the base bCDM either at single addition or in combination to distinguish the indispensable nutrient from non-indispensable ones. Similarly, after single-strain

inoculation followed by incubation, the nutrient or combination of nutrients that supported the growth of the strains was identified. The base formula of the bCDM was then finalized by omitting the carbon and nitrogen sources.

3.3.2 Finalized composition

The finalized composition of the bCDM is listed in Table 4 together with the preparation details. Chemicals were obtained from Sigma (Germany) unless otherwise specified.

Table 4. Finalized preparation of the bCDM.

Chemicals	Concentrations (g/L)
pH of the solution was adjusted to pH 7.5 before sterilization by autoclave (121°C; 15 minutes):	
NaCl	5.0
$K_2HPO_4.3H_2O$ *	2.0
$MgSO_4$	0.2
$MnSO_4.H_2O$ *	0.1
Bromocresol purple	0.03
(Demineralized water)	(1 L)
Sterilization by disk filtration (pore size 0.20 µm; Phenex, the Netherlands):	
L-cysteine.HCl.H_2O	0.5
Thiamin.HCl (for *B. bifidum* only)	0.004
Nicotinic acid (for *B. bifidum* only)	0.002

* Merck, Germany

3.4 Growth experiments

The bCDM and MRS broths were used in the first and second parts of the growth experiments, respectively. The latter medium was employed to investigate the growth-promoting effect of CMP.

In the first part of the growth experiments, bCDM was adjusted to pH 6.6 ± 0.1 before transferring 10 mL into a 15 mL-Greiner tube in duplicate. One group of tubes was supplemented with 10.0 g/L sterile-filtered CMP (Lacprodan CGMP-20; Arla Foods Amba, Denmark), and another group of tubes was added with equal volume of demineralized water as the control. Both groups were inoculated with 10% (v/v) of the PBS+cell suspension. One negative control (non-inoculated) was prepared for each group by adding equal volume of PBS. The tubes were subsequently incubated at 37°C for 38 hours. At the end of the incubation period, the final cell counts were determined by spread-plating as described in the last paragraph of Section 3.2. Additionally, the bound sialic acid contents were quantified as described in Section 3.5.

In the second part of the growth experiments, inocula with lower cell counts were prepared by twice diluting 1 mL of respective PBS+cell suspensions having the OD_{600nm} 0.10-0.35 (Section 3.2) with 99 mL of PBS. MRS broth, prepared as stated on the label, was adjusted to pH 7.0 prior to autoclave. After that, it was supplemented with 0.5 g/L sterile-filtered L-cysteine.HCl monohydrate and adjusted to pH 6.2 ± 0.1 before use. The subsequent steps were similar as in the first part of the growth experiments, except that the tubes in the test group were added with 1.5 g/L of CMP and inoculated with lower cell counts, and finally all tubes were incubated at 37°C for 22 hours. Figure 5 displays the experimental flow chart.

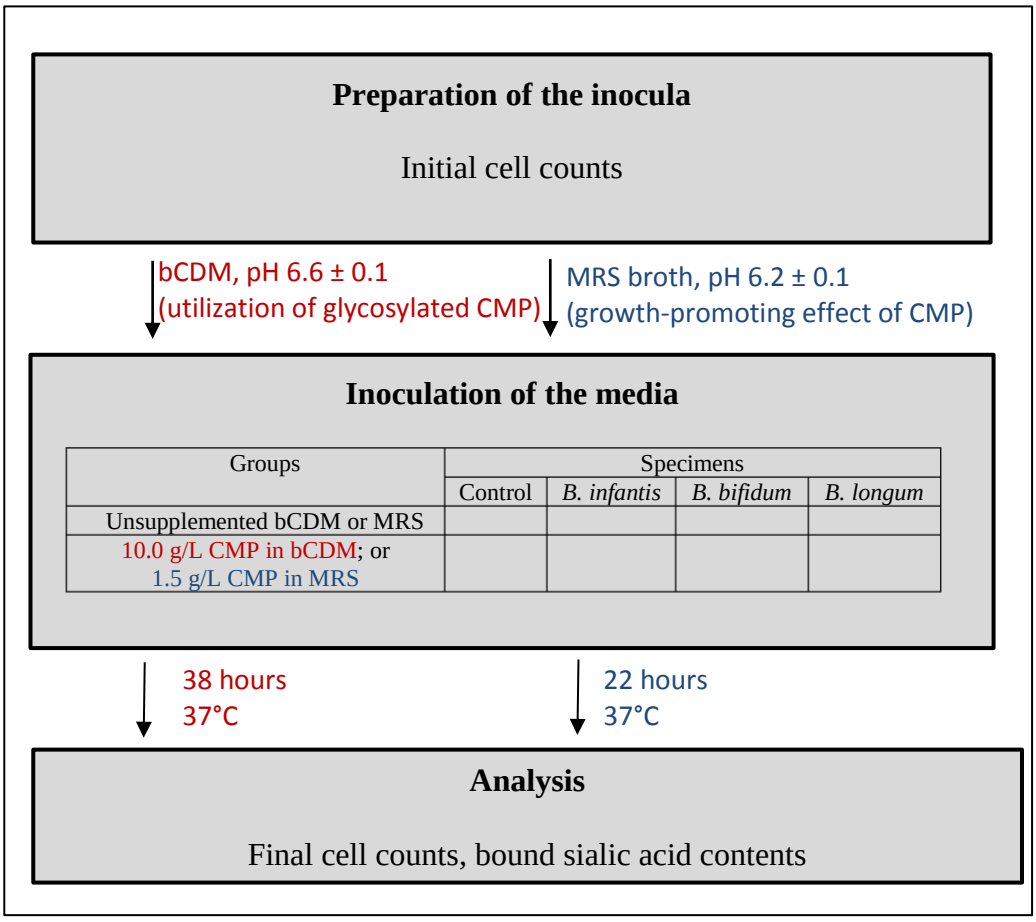


Figure 5. Experimental flow chart.

3.5 Sialic acid quantification by acidic ninhydrin method

The method was adapted from Fukuda *et al.* (2004) with modification. All chemicals were of analytical grade. All samples were analyzed in duplicate.

3.5.1 Preparation of the standard curve

The molecular mass of sialic acid is 309.27 g/mol. A stock standard of 100mM (100 nmol/µL) sialic acid was prepared by dissolving 0.0030 g of pure sialic acid powder (Carbosynth, UK) in 0.1 mL of MilliQ water (purified by Purelab Ultra, Elga, USA). It was kept on ice when in use and stored in a -20°C freezer.

1mM and 10mM sialic acid standard solutions were freshly prepared using the stock standard and MilliQ water as the diluent. From these standard solutions, 1.5mL-Eppendorf tubes were respectively filled with 0, 5, 10, 20, 50, 100, 200, and 500 nmol sialic acid. All tubes were added with 0.2 mL of pure glacial acetic acid (Merck, Germany) and 0.1 mL of acidic ninhydrin reagent. The latter reagent was prepared by dissolving 1.0 g of ninhydrin crystals (Sigma, Germany) in 16 mL of 37% (w/w) hydrochloric acid (AnalaR, the Netherlands) and 24 mL of pure glacial acetic acid.

Subsequently, the Eppendorf tubes were vortexed thoroughly, and heated at 100°C for 10 minutes using a heating block (QBT4, Grant Instruments, UK). The tubes were chilled on ice prior to topping up to exactly 1.0 mL with absolute ethanol (Merck, Germany). The tubes were vortexed immediately before measuring the OD_{470nm} with a spectrophotometer. A standard curve of sialic acid was finally plotted.

3.5.2 Analysis of the samples

At the end of the incubation period of the growth experiments, the tubes were centrifuged at 4700 rpm for 5 minutes at 4°C. 2 mL of each supernatant was mixed with 2 mL of 240 g/L trichloroacetic acid (TCA) (Merck, Germany) and allowed to stand for 30 minutes. The solution was filtered through a disk filter (0.20 µm pore size) before 1 mL of the filtrate and 0.1 mL of 200 g/L phosphotungstic acid hydrate (Sigma, Germany) were filled into an 1.5mL-Eppendorf tube. After a centrifugation at 3500 rpm for 10 minutes at 20°C, the supernatant was gently discarded and 0.6 mL of 95% (v/v) ethanol (prepared from absolute ethanol) was added to the precipitate. Centrifugation was done once again with the same parameters and the supernatant was also gently discarded.

Like the standard solutions, 0.2 mL of pure glacial acetic acid and 0.1 mL of acidic ninhydrin reagent were then added into each tube in which containing precipitates at this stage. Next, the Eppendorf tubes were vortexed thoroughly, heated at 100°C for 10 minutes, chilled on ice, topped up to exactly 1.0 mL with absolute ethanol, and vortexed again. Following that, another centrifugation at 3500 rpm for 5 minutes at 20°C was carried out. The OD_{470nm} of each tube was finally measured with a spectrophotometer. The number of moles (nmol) of sialic acid in the respective tube was obtained by using the equation generated from the standard curve. The bound sialic acid content (g/L) was calculated with Equation 1.

Equation 1:

$$\text{Bound sialic acid content (g/L)} = \frac{n \times M}{V}$$

where,

n = number of moles (nmol); obtained from the equation of the standard curve
M = molecular mass of sialic acid (g/nmol); 3.0927×10^{-7} g/nmol in this case
V = volume of solution (L); 10^{-4} L in this case

3.5.3 Spike test

A CMP assay with the final concentration of 2 g/L was prepared with bCDM and treated the same as described in Section 3.5.2 without the centrifugation step in the very beginning. Before heating at 100°C, 0.05 mL of 1mM sialic acid standard solution (thus, 50 nmol sialic acid) was added into three 'spike' tubes (triplicate). One control was prepared as 'original' tube. The recovery yield (%) was then calculated with Equation 2.

Equation 2:

$$\text{Recovery yield (\%)} = \frac{C_k - C_o}{C_s} \times 100\%$$

where,

C_k = bound sialic acid content of the spiked sample (g/L)
C_o = bound sialic acid content of the original sample (g/L)
C_s = amount of sialic acid intentionally added (g/L)

The percentage of bound sialic acid content in the medium and in the CMP (% w/v) were calculated by Equations 3 and 4, respectively.

Equation 3:

$$\text{\% (w/v) of bound sialic acid in the medium} = \frac{C_{CMP} - C}{V} \times 100\%$$

Equation 4:

$$\% \text{ (w/v) of bound sialic acid in the CMP} = \frac{C_{CMP} - C}{m_i} \times 100\%$$

where,

C_{CMP} = bound sialic acid content in the non-inoculated, CMP-supplemented medium (g/L)
C = bound sialic acid content in the non-inoculated, unsupplemented medium (g/L)
V = volume of solution (mL); 1000 mL in this case
m_i = concentration of the CMP supplemented into the medium (g/L)

3.6 Data and statistical analyses

The mean values and standard deviations of bound sialic acid contents and cell counts were computed using Equations 5 and 6, respectively. The mean values were used for comparison among the specimens and between the groups, whereas the standard deviations were used to determine the precision of the results. One-way analysis of variance (ANOVA) test using SPSS Statistics 22 (IBM, USA) was also performed on the results. The difference was considered significant at the level $p<0.05$.

Equation 5:

$$\text{Mean} = \frac{\Sigma x}{n}$$

Equation 6:

$$\text{Standard deviation} = \sqrt{\frac{\Sigma (x - \bar{x})^2}{n-1}}$$

where,

Σ = the sum of
x = value
$\bar{x}$ = mean value
n = total number of the test samples

4 RESULTS

4.1 Development of a basal chemically-defined medium (bCDM)

A bCDM, which was devoid of carbon and nitrogen sources, was employed in the first part of the growth experiments to obtain reliable results in investigating the utilization of bovine glycosylated CMP by *B. infantis* ATCC 15697, *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707. The formula suggested by Hassinen *et al.* (1951) was used as a reference for the bCDM development in this project as their study contained the most relevant information.

Literature has demonstrated a great heterogeneity of growth requirements among the *Bifidobacterium* spp. For example, *B. adolescentis* SI-30 synthesizes thiamin and nicotinic acid, but *B. adolescentis* E-194a does not synthesize them and indeed requires them for growth (Deguchi *et al.*, 1985). Therefore, to determine the suitability of the formulas, preliminary experiments (an image is provided in Appendix 1) were carried out with potentially indispensable nutrients.

4.1.1 Temporary nitrogen source of the bCDM

A suitable nitrogen source had to be first identified to ensure that the bCDM can support the growth of the three strains. As D-glucose is the most common fermentable carbohydrate by *Bifidobacterium* spp., hence it was temporarily used as the base bCDM (Table 5) alongside with $MgSO_4$, $MnSO_4$ and L-cysteine which are known to support the growth of all bifidobacteria (Whitman *et al.*, 2012). Other chemicals included in the base formula were NaCl (to balance the osmotic pressure between intracellular and extracellular environment), K_2HPO_4 (to act as a buffer), and bromocresol purple (to perceive bifidobacterial growth by detecting pH changes in the solution). The base bCDM was sterilized by autoclave and served as the control.

Table 5. Initial formula of bCDM.

Nutrients/Chemicals	Concentrations (g/L)
D-glucose.H_2O	15.0
NaCl	5.0
$K_2HPO_4.3H_2O$	2.0
L-cysteine.HCl.H_2O	0.5
$MgSO_4$	0.2
$MnSO_4.H_2O$	0.1
Bromocresol purple	0.03

Sterile-filtered nitrogen sources were added into the base bCDM at single addition to distinguish the fermentable from non-fermentable ones, and the result is shown in Table 6. Two ammonium salts were included among the eight nitrogen sources tested because certain strains, for instance *B. adolescentis*, can grow on ammonium salts as a sole nitrogen source (Matteuzzi *et al.*, 1976).

Table 6. Fermentability of eight nitrogen sources by the bifidobacteria.

Nutrients/Chemicals	Concentrations (g/L)	Strains		
		***B. infantis* ATCC 15697**	***B. bifidum* ATCC 29521**	***B. longum* ATCC 15707**
Bacteriological peptone	2.0	+	-	+
Tryptone	2.0	+	-	+
Yeast extract	1.0	+	+	+
Casamino acid	2.0	±	-	+
Sodium caseinate	1.0	-	-	±
Casein hydrolysate (acid)	1.0	±	-	±
Ammonium sulfate	1.0	-	-	-
Ammonium citrate tribasic	1.0	-	-	-

+ : Growth detected as the solution turned yellow;
± : Growth suspected as the solution turned slightly yellowish;
- : No growth detected as the solution remained purple.

Both *B. infantis* ATCC 15697 and *B. longum* ATCC 15707 provided clearly positive results with the supplementation of bacteriological peptone or tryptone into the initial formula of bCDM. This leads to an inference that the substances in the current formula (Table 5) were sufficient to support the growth of these two strains. Bacteriological peptone was chosen as the nitrogen source in the subsequent step of the preliminary experiment since it is relatively more affordable than tryptone. Although all strains could grow very well upon the supplementation of yeast extract, but yeast extract is a

complex substance - it contains carbohydrates, B vitamins and trace minerals. Therefore, it was not included into consideration for further use. Nevertheless, it signified that *B. bifidum* requires additional B vitamins and/or trace minerals for growth.

4.1.2 Other essential nutrients of the bCDM

The subsequent experiment was carried out to identify the essential nutrients required by *B. bifidum* ATCC 29521 for growth. The composition of the base bCDM used at this stage is shown in Table 7. It was sterilized by autoclave and served as the control.

Table 7. Base formula of bCDM used to identify the essential nutrients for *B. bifidum* ATCC 29521.

Nutrients/Chemicals	Concentrations (g/L)
D-glucose.H_2O	15.0
NaCl	5.0
Bacteriological peptone	2.0
$K_2HPO_4.3H_2O$	2.0
L-cysteine.HCl.H_2O	0.5
$MgSO_4$	0.2
$MnSO_4.H_2O$	0.1
Bromocresol purple	0.03

Six B vitamins and four nucleobases were added into the base bCDM at single addition by sterile-filtration. However, each of them did not support the growth of *B. bifidum*. Thus, the vitamins and nucleobases were added in combination. The results are displayed in Table 8.

Table 8. Effect of the addition of nutrients on the bifidobacteria.

Nutrients	Strains		
	***B. infantis* ATCC 15697**	***B. bifidum* ATCC 29521**	***B. longum* ATCC 15707**
0.004 g/L thiamin	-	-	+
0.002 g/L nicotinic acid	-	-	+
A: 0.004 g/L thiamin + 0.002 g/L nicotinic acid	-	+	+
B: 0.002 g/L riboflavin + 0.002 g/L biotin + 0.002 g/L cyanocobalamin + 0.002 g/L calcium pantothenate	+	-	+
A + B	+	+	+
A + 0.03 g/L uracil + 0.03 g/L xanthine + 0.015 g/L adenine + 0.003 g/L guanine	-	+	+

+ : Growth detected as the solution turned yellow;
- : No growth detected as the solution remained purple.

Notably, both thiamin and nicotinic acid were supportive for the growth of *B. bifidum*. The addition of riboflavin, calcium pantothenate, biotin, cyanocobalamin, uracil, xanthine, adenine, or guanine did not have major effect on its growth if both thiamin and nicotinic acid were present. In contrary, *B. infantis* could not grow in the presence of thiamin, nicotinic acid, or both; whereas *B. longum* can grow well with or without any additional nutrients.

4.1.3 Finalized formula of the bCDM

The base formula of the bCDM was then finalized by omitting the carbon and nitrogen sources (Table 9). As described in Section 3.4, sterilized base bCDM was used as the control, whereas the test group was supplemented with 10.0 g/L sterile-filtered CMP. Since the addition of thiamin and nicotinic acid seemed to negatively affect the growth of *B. infantis* ATCC 15697, the vitamins were added only for the tubes inoculated by *B. bifidum* ATCC 29521.

Table 9. Overview of the base formula of the bCDM.

Nutrients/Chemicals	Concentrations (g/L)
NaCl	5.0
$K_2HPO_4.3H_2O$	2.0
L-cysteine.HCl.H_2O	0.5
$MgSO_4$	0.2
$MnSO_4.H_2O$	0.1
Bromocresol purple	0.03
Thiamin.HCl (for *B. bifidum* only)	0.004
Nicotinic acid (for *B. bifidum* only)	0.002
(Demineralized water)	(1 L)

4.2 Final cell counts in the growth experiments

4.2.1 Slower microbial death in bCDM+CMP

Significant decrement of cell counts was found in all inoculated media at the end of the incubation period instead of the expected growth (Table 10; Appendices 2-3). No growth was also revealed by the persistence of the purple color in the inoculated tubes.

Table 10. Cell counts in bCDM+CMP and bCDM.

Strains	Cell counts (cfu/mL)			
	Start of incubation time (0 h)		End of incubation time (38 h)	
	bCDM+CMP	bCDM	bCDM+CMP	bCDM
B. infantis ATCC 15697	2.44×10^7	2.44×10^7	1.05×10^4 [a]	2.00×10^2 [b]
B. bifidum ATCC 29521	2.06×10^6	2.06×10^6	9.55×10^4 [a]	8.65×10^3 [b]
B. longum ATCC 15707	2.07×10^7	2.07×10^7	1.37×10^6 [a]	3.10×10^4 [b]

[a, b] Different superscript letters in the same row are significantly different ($p<0.05$) from each other.

4.2.2 Promoted microbial growth in MRS+CMP

MRS was used in the second part of the growth experiments to allow normal growth of the strains. Bifidobacterial growth in MRS with or without CMP supplementation was compared (Table 11) to study about the growth-promoting effect of CMP. The fold-change of each strain is defined by the ratio of final cell counts in CMP-supplemented broth to unsupplemented broth.

Table 11. Fold-changes of the strains in MRS+CMP and MRS.

Strains	Cell counts (cfu/mL)				Fold-changes (i.e. ratio of final cell counts $\frac{\text{MRS+CMP}}{\text{MRS}}$)
	Start of incubation time (0 h)		End of incubation time (22 h)		
	MRS+CMP	MRS	MRS+CMP	MRS	
B. infantis ATCC 15697	8.00×10^2	8.00×10^2	6.75×10^7	4.10×10^7	1.7
B. bifidum ATCC 29521	9.00×10^2	9.00×10^2	8.25×10^8	5.43×10^8	1.5
B. longum ATCC 15707	7.56×10^6	7.56×10^6	9.27×10^8	8.15×10^8	1.1

All strains reached higher cell counts in MRS+CMP compared to MRS. This result was expected because CMP represents additional nitrogen and carbon sources in the MRS medium. The fold-changes of *B. infantis* grew on MRS+CMP seemed to be slightly higher than *B. bifidum* and *B. longum*, though the difference in fold-changes was not significant (Appendix 4).

Additional MRS agar plates of each inoculum were incubated aerobically to check for possible microbial contamination. All inocula had never been contaminated by other microbes, probably due to competitive exclusion. Interestingly, *B. longum* grew aerobically at 37^{o}C but required twice as long incubation period as compared to the plates that were incubated in the anaerobic environment. This result was consistent with the observation of the culture tubes (Appendix 5) where *B. longum* was spread throughout the tube containing 10 mL MRS broth. Conversely, *B. bifidum* and *B. infantis* did not grow on the plates incubated aerobically and accumulated at the bottom

of the culture tubes where oxygen concentration was the lowest. Thus, *B. longum* ATCC 15707 is an aerotolerant microbe, most probably due to the presence of enzymes that can repair oxidative damage like those present in *B. longum* NCC2705 (Schell *et al.*, 2002), while the other two strains are strict anaerobes.

4.3 Final bound sialic acid contents in the growth experiments

Bound sialic acid contents decreased in inoculated bCDM+CMP and MRS+CMP after incubation (Figure 6). The largest decrement was found in the media inoculated by *B. infantis*. Contrarily, the smallest decrement was obtained in the media inoculated by *B. longum*. This implies that the sialic acid residues from the glycosylated CMPs were cleaved and utilized the most by *B. infantis*, followed by *B. bifidum* and the least by *B. longum*. The standard curve of sialic acid can be found in Appendix 6.

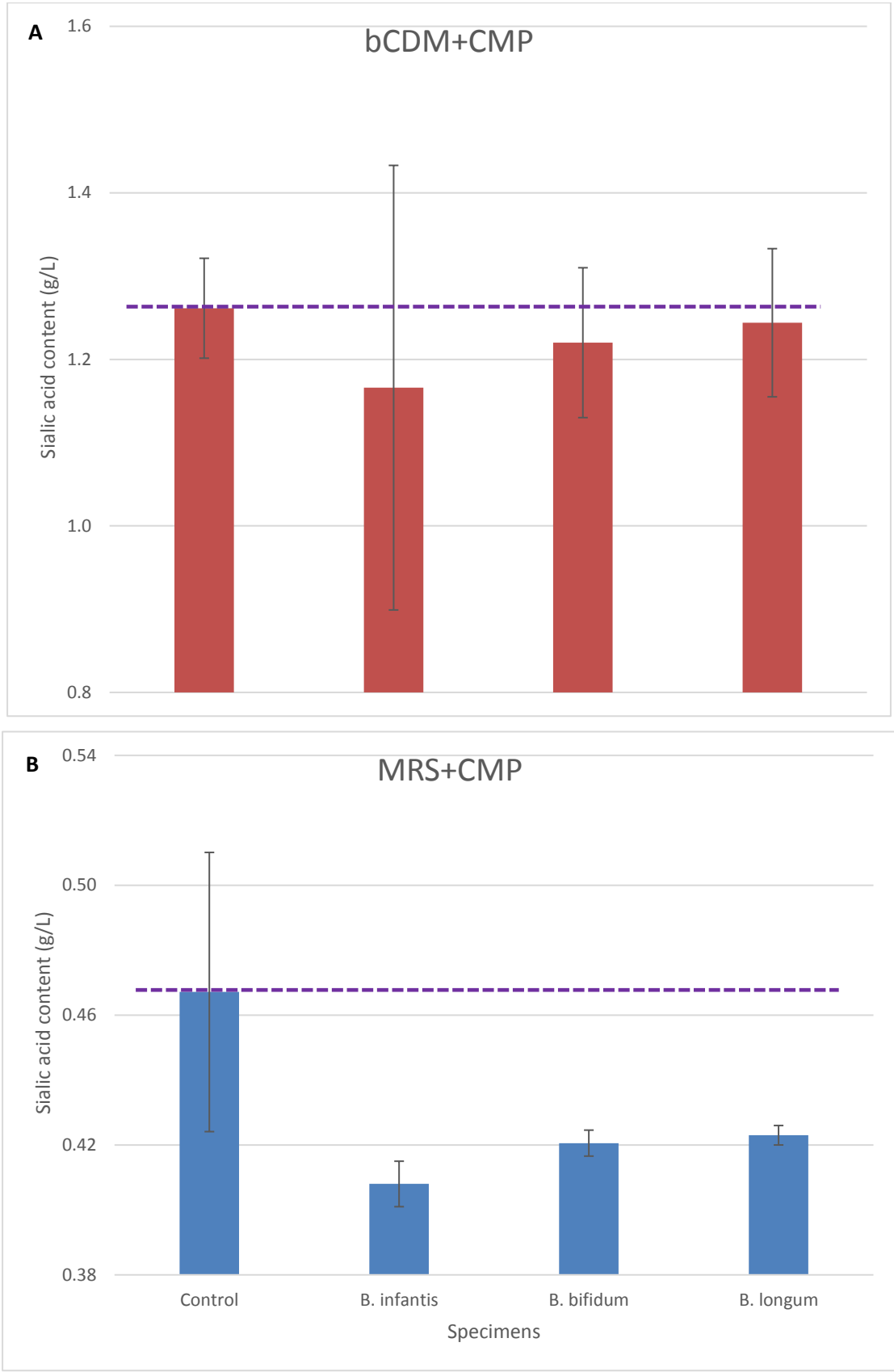


Figure 6. Bound sialic acid contents in the media after designated hours of incubation. Note that the vertical axes scales are set at different intervals and do not begin at zero. Horizontal dotted lines indicate the levels of bound sialic acid in the negative controls. Error bars represent standard deviations of each mean value. No significant difference is found among the specimens in the same medium (Appendices 7 and 8).

The percentage differences of bound sialic acid contents between inoculated tubes and the respective controls can be found in Table 12. Generally, higher percentage of reduction in bound sialic acid contents is found in MRS+CMP than in bCDM+CMP, though no significant difference is present (Appendix 9).

Table 12. Differences of sialic acid contents between bCDM+CMP and MRS+CMP with the respective control.

Media	**Differences of bound sialic acid contents with the control**	**Strains**		
		***B. infantis* ATCC 15697**	***B. bifidum* ATCC 29521**	***B. longum* ATCC 15707**
bCDM+CMP	g/L	-0.095 ± 0.207	-0.041 ± 0.029	-0.017 ± 0.029
	%	-7.6	-3.3	-1.4
MRS+CMP	g/L	-0.059 ± 0.035	-0.047 ± 0.039	-0.045 ± 0.040
	%	-12.6	-10.0	-9.5

4.4 Reliability of acidic ninhydrin method

A spike test was carried out to evaluate the accuracy of the acidic ninhydrin method in determining the bound sialic acid content in the media. Initially, a CMP assay of 2 g/L was spiked by 50 nmol sialic acid before the precipitation by trichloroacetic acid (TCA). The results showed similar sialic acid contents between spiked and non-spiked samples. This leads to a conclusion that spiking at this stage was not appropriate as all free sialic acids will be lost alongside with the discard of the unwanted supernatant succeeding the TCA-precipitation step. Therefore, the CMP assay was spiked by 50 nmol sialic acid after TCA-precipitation step but before heating. An acceptable mean recovery is between 90 to 110% (Liang *et al.*, 2016). The result shown in Table 13 implies that the method to measure sialic acid, as employed by Fukuda *et al.* (2004), had been carried out accurately in this project.

Table 13. Recovery yield of the spike test.

No.	Sialic acid contents (g/L)			Recovery yield (%)
	Spiked sample	Original sample	Standard added	
1	0.326	0.161	0.155	106.5
2	0.315	0.161	0.155	99.4
3	0.335	0.161	0.155	112.3
			Mean recovery (%)	106.1

The bound sialic acid content in the CMP powder was calculated (Table 14) and compared with the actual value to assess the effectiveness of the acid precipitation.

Table 14. Bound sialic acid contents in the media and in the CMP.

	Nutrient-rich medium		Nutrient-depleted medium	
	MRS+CMP	MRS	bCDM+CMP	bCDM
Concentration of CMP (g/L)	1.5	0.0	10.0	0.0
Bound sialic acid contents (g/L)	0.467 ± 0.042	0.324 ± 0.077	1.261 ± 0.060	0.010 ± 0.005
Percentage (w/v) of bound sialic acid in the CMP (%)	9.5		12.5	
Percentage (w/v) of bound sialic acid originating from the CMP in the medium (%)	0.01		0.13	

The calculation of 9.5% (w/v) of bound sialic acid in the CMP in MRS+CMP is close to the actual value of 9% (w/w) (Jers *et al.*, 2014). This could imply that the acid precipitation was effective. However, the supplier stated that the sialic acid content may vary and a typical value is 4-6% (w/w). Therefore, relatively lower value obtained by the supplier might due to the different methods used for the sialic acid quantification.

In short, acidic ninhydrin method represents a reliable and simple means to quantify sialic acid.

5 DISCUSSIONS

5.1 Growth requirements of the strains

A bCDM, which was devoid of carbon and nitrogen sources, was employed in the first part of the growth experiments to obtain reliable results in investigating the utilization of bovine glycosylated CMP by the strains.

Notably, the result (Table 8) suggested that *B. bifidum* ATCC 29521 requires both thiamin and nicotinic acid to grow. The reason may be that this strain cannot synthesize the vitamins for its energy metabolism, unlike *B. adolescentis* SI-30 (Deguchi *et al.*, 1985). Conversely, the addition of thiamin, or nicotinic acid, or both into the base bCDM without the presence of other B vitamins seemed to exert an inhibition on the growth of *B. infantis* ATCC 15697. It is somewhat contradictory to the finding that this strain can synthesize thiamin and nicotinic acid, even with the addition of exogenous thiamin (Deguchi *et al.*, 1985). One possible reason to explain the observed growth inhibition on *B. infantis* ATCC 15697 could be due to the elevated level of thiamin (0.004 g/L) added into the bCDM in this project, as compared to the highest concentration of 0.0002 g/L used by Deguchi *et al.* (1985). Although thiamin toxicity in bifidobacteria is not known to date, but excessive thiamin has shown to alter the enzymatic activities, and consequently inhibited the growth of *Saccharomyces carlsbergensis* ATCC 9080 in a vitamin B_6-free medium (Nakamura *et al.*, 1982).

Literature has shown that *Bifidobacterium* spp. may grow on a simplified medium comprised of a fermentable carbohydrate, ammonium salt, buffers, minerals, cysteine as well as B vitamins (Hassinen *et al.*, 1951). L-cysteine is required by most *Bifidobacterium* spp. for proper formation of proteins, in addition to lower the redox potential of the media (Ferrario *et al.*, 2015). Thus, it is confirmed to be indispensable for *Bifidobacterium* spp. The minerals mentioned by Hassinen *et al.* (1951) refers to the combination of $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, NaCl, and $MnSO_4.H_2O$, since the individual elimination of any of the minerals did not decrease the bifidobacterial growth. While magnesium is essential for the formation of cell wall in Gram-positive bacteria and for the activation of certain enzymes, manganese has the similar functions but to a lesser activity (Webb, 1951). This may be the reason why single-removal of the minerals

by Hassinen *et al.* (1951) did not affect the bifidobacterial growth. Hence, $FeSO_4.7H_2O$ is not required for bifidobacterial growth as proven in this project, and possibly $MnSO_4.H_2O$ as well.

In short, the minimal medium allowing the growth of *B. infantis* ATCC 15697 and *B. longum* ATCC 15707 could be as simple as a fermentable carbohydrate, a protein source, buffers, $MgSO_4$, and L-cysteine. Whereas *B. bifidum* ATCC 29521 requires thiamin and nicotinic acid in addition to the abovementioned nutrients/chemicals.

5.2 Better microbial survival in bCDM+CMP

In the first part of the growth experiments of this project, there was a drop in the cell counts in bCDM+CMP (Table 10). Microbial death, instead of cell proliferation, was most probably because the carbon source from glycosylated CMPs (0.13% w/v sialic acid in the bCDM+CMP; Table 14) was too scarce and quickly exhausted in the medium. Literature has shown that at least 0.4% (w/v) HMOs in a semi-synthetic MRS medium (without a carbohydrate source) is required for the growth of *B. infantis* ATCC 15697 (Locascio *et al.*, 2007).

The slower death of all three strains in CMP-supplemented bCDM may indicate that it can act as the sole source of carbon and nitrogen in the medium for cellular metabolism. Thus, it is ascertained that the glycan chains attached to the glycosylated CMPs were utilized to some extent by all three strains via enzymatic degradation in a sequence-specific manner (Section 5.2.1).

5.2.1 Enzymatic degradation of *O*-linked glycans

The most abundant (56.0%) type of chain that attaches to a CMP is tetrasaccharides (Saito & Itoh, 1992). The removal of glycan chains from a glycoprotein occurs in a specific sequence (Figure 7). For the full utilization of the glycan chain, sialic acid residues must be removed prior to exposing the galacto-*N*-biose (GNB; it is also known as “Core 1 structure”) for degradation (Jiskoot & Crommelin, 2005).

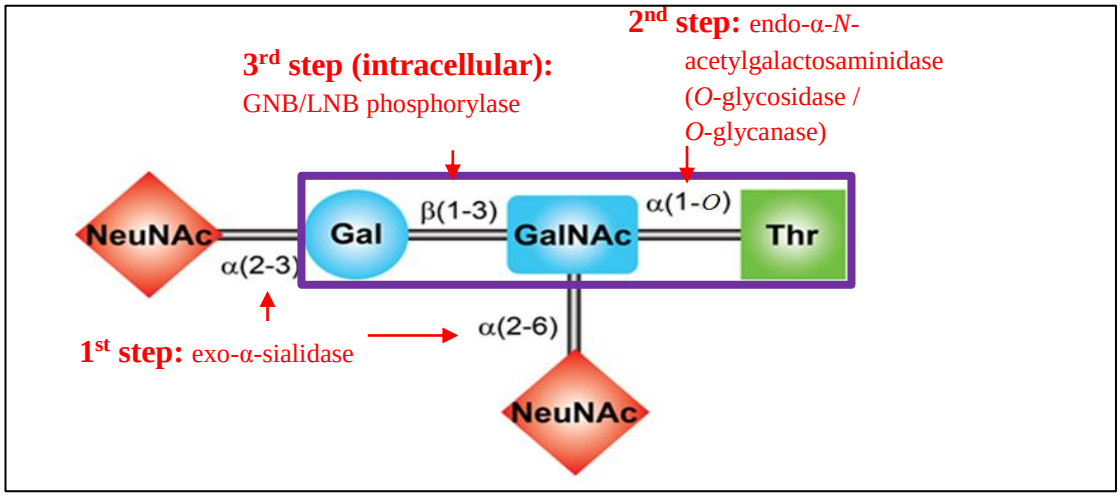


Figure 7. Enzymatic degradation of a tetrasaccharides chain in O-linked glycosylation. The purple box depicts a GNB. Hydrolysis steps by respective enzymes are marked in red.

5.2.2 Sialic acid cleavage

Exo-α-sialidase is a glycoside hydrolase that cleaves α-glycosidic bonds of terminal sialic acid residues in oligosaccharides and glycoconjugates (Kiyohara *et al.*, 2011).

This enzyme is produced by *B. infantis* ATCC 15697 (Kiyohara *et al.*, 2011; Locascio *et al.*, 2007). With the aid of the binding proteins at the cell surface, the liberated sialic acid residues then transverses through ATP-binding cassette (ABC) transporters into the cell. Sialic acids are then further processed by the intracellular enzymes and ultimately generate energy in the cell (Appendix 10A) (Garrido *et al*, 2012; Sela *et al.*, 2008).

B. bifidum JCM1255 (same as ATCC 29521) demonstrated sialic acid-degrading activity in the studies carried out by Dank (2016), Ward *et al.* (2007), and Idota *et al.* (1994). Despite that, this strain does not produce exo-α-sialidase as *B. bifidum* JCM1254 and *B. bifidum* JCM7004 do (Kiyohara *et al.*, 2011). The sialic acid residues associated to a glycoprotein are released by a yet-to-be-characterized enzyme (putative sialidase) in *B. bifidum* ATCC 29521 but the generated free sialic acids are left unutilized (Milani *et al.*, 2015; Ward *et al.*, 2007).

Similarly, exo-α-sialidase is not produced by *B. longum* JCM1217 (same as ATCC 15707) (Kiyohara *et al.*, 2011), but Dank (2016) and Idota *et al.* (1994) had found sialic

acid-degrading activity by this strain as well. Furthermore, Locascio *et al.* (2007) acknowledged a minimal and nonspecific sialidase activity in another closely related strain, which is *B. longum* DJO10A, despite it does not have exo-α-sialidase.

5.2.3 Galacto-*N*-biose (GNB) cleavage

Endo-α-*N*-acetylgalactosaminidase is a glycoside hydrolase that cleaves α-glycosidic bonds linking Gal*N*Ac-containing disaccharides with Ser or Thr residues, resulting in the release of the *O*-linked glycan chains from its conjugated peptide backbone (Koutsioulis *et al.*, 2008).

The enzyme is present in *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 as an extracellular enzyme (Fujita *et al.*, 2005). After an intact GNB binds to the binding protein (in this case is known as "galacto-*N*-biose/lacto-*N*-biose I-binding protein" / GLBP) on the cell surface, endo-α-*N*-acetylgalactosaminidase removes it from its connected peptide backbone (Suzuki *et al.*, 2008). The ABC transporter subsequently transverses the GNB into the cell. Finally, intracellular enzymes degrade it and utilize the monosaccharides for cellular metabolism (Appendix 10B) (Bruyn *et al.*, 2013; Kitaoka *et al.*, 2005).

Contrarily, endo-α-*N*-acetylgalactosaminidase is absent in *B. infantis* (Fujita *et al.*, 2005). The strain does not consume *O*-linked glycans but only *N*-linked glycans (Karav *et al.*, 2016). Despite that, it imports all oligosaccharides into the cell via specific binding proteins and ABC transporters, then degrades the substrates intracellularly (Asakuma *et al.*, 2011). The reason behind this is probably due to the genome adaptation of this strain to their habitat (human infant), and free oligosaccharides are primarily present in human milk (Ward *et al.*, 2007).

5.2.4 Utilization of the glycosylated CMP

As a summary, the ability to utilize glycosylated CMP is strain-specific. *B. infantis* ATCC 15697 survived longer in bCDM+CMP by cleaving and utilizing the sialic acid

residues attached to the glycosylated CMPs, but it is theoretically not able to utilize the other glycans due to the lack of extracellular endo-α-*N*-acetylgalactosaminidase. Whereas, the presence of putative sialidases in *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707, which has not been characterized yet, assisted in their survival in bCDM+CMP. Following the cleavage of terminal sialic acid residues from the glycan chains, both strains utilized the remaining intact GNB as a carbon source for their metabolism. It might also be that *B. longum* degraded the sialylated glycan chains without prior removal of the sialic acid residues, as demonstrated for sialyllacto-*N*-tetraose (Odamaki *et al.*, 2015).

5.3 Growth-promoting effect of CMP on the tested strains

MRS was used in the second part of the growth experiments in this project to allow normal growth of the strains. All strains reached higher cell counts in MRS+CMP than in MRS (Table 11). This result was also consistent with the study which demonstrated a shortened lag phase and a higher final cell count of *B. bifidum* CCDM94 attributed to the supplementation of CMP in the growth media (Cicvárek *et al.*, 2010).

This result was expected because CMP consists of a short polypeptide which is preferably used by *Bifidobacterium* spp. in comparison to large protein molecules (Robitaille, 2013). Additionally, the *O*-linked glycan chains attached to the CMPs are utilizable by the strains (Section 5.2). Although endo-α-*N*-acetylgalactosaminidase is theoretically absent in *B. infantis* in which results in the inability to degrade GNBs extracellularly, the abundance of carbon sources in MRS had probably masked that disadvantage over the other two strains.

The fold-changes of *B.* infantis, B. *bifidum* and *B. longum* were 1.7, 1.5, and 1.1, respectively, as displayed in Table 11. *B. longum* had the lowest fold-change probably due to its inoculation of relatively higher initial cell counts than the other two strains. Low inocula were intended in this part of the growth experiments to intensify the growth-promoting effect of CMP (Brück *et al.*, 2006). It has been shown that the bifidobacterial lag phase is inversely correlated with the initial cell count (Cicvárek *et al.*, 2010). This means that a high inoculum reaches the maximal cell count considerably quicker than using a low inoculum in the same nutrient-rich medium.

5.4 Decrease of bound sialic acid contents in the inoculated media

The largest decrement of bound sialic acid contents was found in the media inoculated by *B. infantis* (Figure 6). This result was expected as it produces exo-α-sialidase which cleaves off terminal sialic acid residues from a glycan chain (Kiyohara *et al.*, 2011). Whereas the loss of sialic acid in the media inoculated by *B. bifidum* and *B. longum* correlates with the better bifidobacterial survival in bCDM+CMP, therefore strengthens the possibility of sialidases in these two strains.

Higher percentage of reduction in bound sialic acid contents is found in all inoculated MRS+CMP than in bCDM+CMP (Table 12). This is most probably due to the presence of other carbon sources in MRS which had supported the growth of the bifidobacteria, and induced the synthesis of glycoside hydrolases including sialidases. Consequently, more sialic acid residues were released from the glycan chains of the glycosylated CMPs in MRS+CMP than in bCDM+CMP. This was consistent with the report by Turroni *et al.* (2010) where *B. bifidum* PRL2010 produced ten times more endo-α-*N*-acetylgalactosaminidase when growing on HMOs than growing on lactose as the sole carbon source.

6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

B. infantis ATCC 15697 can cleave and utilize sialic acids, but not other glycans, that are attached to the glycosylated CMPs; while both *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 can cleave terminal sialic acid residues, followed by the utilization of the glycans attached to the glycosylated CMPs other than sialic acids. This indicates that the hypothesis of "utilize all glycans attached to the CMP except sialic acids" by *B. bifidum* ATCC 29521 can be accepted; whereas the hypotheses of 'a full utilization of glycosylated CMP' by *B. infantis* ATCC 15697 and 'a minimal utilization of

glycosylated CMP' by *B. longum* ATCC 15707 should be rejected. It is therefore postulated that both *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 can produce sialidases to cleave sialic acid residues, which have not been characterized yet to date. This is supported by the slower death of all three strains in bCDM+CMP as compared to bCDM, and the reduction in bound sialic acid contents in inoculated media.

Additionally, bovine CMP exerted a similar growth-promoting effect on all the strains because CMP consists of a short polypeptide which is preferably used by bifidobacteria. This conclusion was drawn based on the final cell counts of the three strains that were higher in MRS+CMP than in MRS. The growth-promoting effect of CMP on the strains did not differ significantly though, unlike what had been hypothesized "enhance the growth (with the effect intensity in descending order) of *B. infantis*, *B. bifidum*, and *B. longum*". Moreover, the presence of other carbon sources in MRS might induce the synthesis of glycoside hydrolases in the strains, particularly sialidases, therefore leading to a higher percentage of reduction in bound sialic acid contents in MRS+CMP than in bCDM+CMP.

From an industrial point of view (for example, functional ingredients manufacturers), it is feasible to add bovine CMP, which is largely found in the rennet whey during cheese-making, into the bifidobacterial growth medium to enhance their growth.

6.2 Recommendations

Further research on the putative sialidases in *B. bifidum* ATCC 29521 and *B. longum* ATCC 15707 is warranted. This can be done by identifying the potential genes, cloning the genes, and characterizing the recombinant proteins (an example of method by Kiyohara *et al.*, 2012). Additionally, the use of microplate reader is highly recommended for the research on bacteria. It not only consumes less materials, but also enables the monitoring of bacterial growth in real time, thus more accurate growth curve can be obtained (Brewster, 2003).

The determination of free sialic acid is also recommended so that it is possible to distinguish the ability between the cleavage and the utilization of sialic acid by the strains. Alternatively, it may be useful to culture the strains in ^{13}C-labelled free sialic

acid (an example of method by Young *et al.*, 2015) to learn the metabolism of free sialic acids by the strains.

Apart from that, development of a minimal medium could be done as inferred in the last paragraph of Section 5.1. A variation to the parameters used in the growth experiments of this project could also be done in future works. Examples are extending the incubation period to several days to obtain the complete growth curves of the strains, supplementing the media with different concentrations of CMP to determine its effect on the bifidobacterial growth, as well as co-inoculating *B. infantis* and *B. bifidum* into CMP-supplemented MRS to scrutinize its growth-promoting effect.

BIBLIOGRAPHY

Abd El-Salam, M.H., El-Shibiny, S., & Buchheim, W. (1996). Characteristics and potential uses of the casein macropeptide. *International Dairy Journal* 6 (4), 327-341.

Asakuma, S., Hatakeyama, E., Urashima, T., Yoshida, E., Katayama, T., Yamamoto, K., Kumagai, H., Ashida, H., Hirose, J., & Kitaoka, M. (2011). Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. *Journal of Biological Chemistry* 286 (40), 34583-34592.

Azuma, N., Yamauchi, K., & Mitsuoka, T. (1984). Bifidus growth-promoting activity of a glycomacropeptide derived from human κ-casein. *Agricultural and Biological Chemistry* 48 (8), 2159-2162.

Bao, Z., Sun, S., Li, J., Chen, X., Dong, S., & Ma, H. (2006). Direct identification of tryptophan in a mixture of amino acids by the naked eye. *Angewandte Chemie International Edition* 45 (40), 6723-6725.

Bernini, L.J., Simão, A.N., Alfieri, D.F., Lozovoy, M.A., Mari, N.L., de Souza, C.H., Dichi, I., & Costa, G.N. (2016). Beneficial effects of *Bifidobacterium lactis* on lipid profile and cytokines in patients with metabolic syndrome: A randomized trial. Effects of probiotics on metabolic syndrome. *Nutrition* 32 (6), 716-719.

Bijl, E. (2014). *Natural variation in casein composition of milk*. Unpublished PhD dissertation, Wageningen University.

Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., & Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* 4 (6), 1633-1649.

Bode, L. (2009). Human milk oligosaccharides: Prebiotics and beyond. *Nutrition Reviews* 67 (Suppl. 2), S183-S191.

Bouhallab, S., Favrot, C., & Maubois, J.L. (1993). Growth-promoting activity of tryptic digest of caseinomacropeptide for *Lactococcus lactis* subsp *lactis. Le Lait* 73 (1), 73-77.

Brewster, J.D. (2003). A simple micro-growth assay for enumerating bacteria. *Journal of Microbiological Methods* 53 (1), 77-86.

Brück, W.M., Redgrave, M., Tuohy, K.M., Lönnerdal, B., Graverholt, G., Hernell, O., & Gibson, G.R. (2006). Effects of bovine α-lactalbumin and casein glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term infants. *Journal of Pediatric Gastroenterology and Nutrition* 43 (5), 673-679.

Bruyn, F.D., Beauprez, J., Maertens, J., Soetaert, W., & Mey, M.D. (2013). Unraveling the leloir pathway of *Bifidobacterium bifidum*: Significance of the uridylyltransferases. *Applied and Environmental Microbiology* 79 (22), 7028-7035.

Challa, A., Rao, D.R., Chawan, C.B., & Shackelford, L. (1997). *Bifidobacterium longum* and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis* 18 (3), 517-521.

Cheikhyoussef, A., Cheikhyoussef, N., Chen, H., Zhao, J., Tang, J., & Zhang, H., & Chen, W. (2010). A new bacteriocin produced by *Bifidobacterium infantis* BCRC 14602: Purification and partial amino acid sequence. *Food Control* 21 (5), 746-753.

Chen, T.R., Wei, Q.K., & Chi, Z.X. (2011). Effect of oligosaccharides and isoflavones aglycones in defatted soy meal fermented by *Lactobacillus paracasei* and *Bifidobacterium longum. African Journal of Microbiology Research* 5 (15), 2011-2018.

Cicvárek, J., Čurda, L., Elich, O., Dvořáková, E., & Dvořák, M. (2010). Effect of caseinomacropeptide concentrate addition on the growth of bifidobacteria. *Czech Journal of Food Sciences* 28 (6), 485-494.

Coakley, M., Ross, R.P., Nordgren, M., Fitzgerald, G., Devery, R., & Stanton, C. (2003). Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *Journal of Applied Microbiology* 94 (1), 138-145.

Coolbear, K.P., Elgar, D.F., & Ayers, J.S. (1996). Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *International Dairy Journal* 6 (11-12), 1055-1068.

Coppa, G.V., Bruni, S., Morelli, L., Soldi, S., & Gabrielli, O. (2004). The first prebiotics in humans: Human milk oligosaccharides. *Journal of Clinical Gastroenterology* 38 (Suppl. 2), S80-S83.

Dank, A. (2016). *Enzymatic degradation of oligosaccharides from high and low glycosylated caseinate by probiotic bacteria*. Unpublished BSc dissertation, Wageningen University.

Dasgupta, A. (2015). *Alcohol and Its Biomarkers: Clinical Aspects and Laboratory Determination*, pp. 225-226. San Diego: Elsevier.

Deguchi, Y., Morishita, T., & Mutai, M. (1985). Comparative studies on synthesis of water-soluble vitamins among human species of bifidobacteria. *Agricultural and Biological Chemistry* 49 (1), 13-19.

Delfour, A., Jollès, J., Alais, C., & Jollès, P. (1965). Caseino-glycopeptides: Characterization of a methionin residue and of the N-terminal sequence. *Biochemical and Biophysical Research Communications* 19 (4), 452-455.

Desjardins, M.L., & Roy, D. (1990). Growth of bifidobacteria and their enzyme profiles. *Journal of Dairy Science* 73 (2), 299-307.

Donkor, O.N., Henriksson, A., Vasiljevic, T., & Shah, N.P. (2007). α-Galactosidase and proteolytic activities of selected probiotic and dairy cultures in fermented soymilk. *Food Chemistry* 104 (1), 10-20.

Duranti, S., Milani, C., Lugli, G.A., Turroni, F., Mancabelli, L., Sanchez, B., Ferrario, C., Viappiani, A., Mangifesta, M., Mancino, W., Gueimonde, M., Margolles, A., van Sinderen, D., & Ventura, M. (2015). Insights from genomes of representatives of the human gut commensal *Bifidobacterium bifidum*. *Environmental Microbiology* 17 (7), 2515–2531.

Dziuba, J. & Minkiewicz, P. (1996). Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's κ-casein. *International Dairy Journal* 6 (11-12), 1017-1044.

European Commission (2008). Commission Regulation (EC) No 273/2008 of 5 March 2008. *Official Journal of the European Union L 088*. pp.68-80.

Eurostat (2017). Milk and Milk Product Statistics. Retrieved from <http://ec.europa.eu/eurostat/statistics-explained/index.php/Milk_and_milk_product_statistics#Further_Eurostat_information> on April 12, 2017.

Feleszko, W., Jaworska, J., Rha, R.D., Steinhausen, S., Avagyan, A., Jaudszus, A., Ahrens, B., Groneberg, D.A., Wahn, U., & Hamelmann, E. (2007). Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma. *Clinical and Experimental Allergy* 37 (4), 498-505.

Ferrario, C., Duranti, S., Milani, C., Mancabelli, L., Lugli, G.A., Turroni, F., Mangifesta, M., Viappiani, A., Ossiprandi, M.C., van Sinderen, D., & Ventura, M. (2015). Exploring amino acid auxotrophy in *Bifidobacterium bifidum* PRL2010. *Frontiers in Microbiology* 6, 1331.

Fiat, A.M., Chevan, J., Jollès, P., de Waard, P., Vliegenthart, J.F.G., Piller, F., & Cartron, J.P. (1988). Structural variability of the neutral carbohydrate moiety of cow colostrum κ-casein as a function of time after parturition. *European Journal of Biochemistry* 173 (2), 253-259.

Fujita, K., Oura, F., Nagamine, N., Katayama, T., Hiratake, J., Sakata, K., Kumagai, H., & Yamamoto, K. (2005). Identification and molecular cloning of a novel glycoside hydrolase family of core 1 type *O*-glycan-specific endo-α-*N*-acetylgalactosaminidase from *Bifidobacterium longum*. *Journal of Biological Chemistry* 280 (45), 37415-37422.

Fukuda, S., Roig, S., & Prata, L. (2004). Correlation between acidic ninhydrin and HPLC methods to evaluate fraudulent addition of whey in milk. *Le Lait* 84 (5), 501-512.

Garrido, D., Ruiz-Moyano, S., & Mills, D.A. (2012). Release and utilization of N-acetyl-D-glucosamine from human milk oligosaccharides by *Bifidobacterium longum* subsp. *infantis*. *Anaerobe* 18 (4), 430-435.

Ghazvini, R.D., Kouhsari, E., Zibafar, E., Hashemi, S.J., Amini, A., & Niknejad, F. (2016). Antifungal activity and aflatoxin degradation of *Bifidobacterium bifidum* and *Lactobacillus fermentum* against toxigenic *Aspergillus parasiticus. Open Microbiology Journal* 10 (1), 197-201.

Gill, H.S., Rutherfurd, K.J., Prasad, J., & Gopal, P.K. (2000). Enhancement of natural and acquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019). *British Journal of Nutrition* 83 (2), 167-176.

Gill, H.S., Rutherfurd, K.J., & Cross, M.L. (2001). Dietary probiotic supplementation enhances natural killer cell activity in the elderly: An investigation of age-related immunological changes. *Journal of Clinical Immunology* 21 (4), 264-271.

Gomes, A.M.P., & Malcata, F.X. (1999). *Bifidobacterium* spp. and *Lactobacillus acidophilus*: Biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science and Technology* 10 (4-5), 139-157.

Gottschalk, A. (1960). *The Chemistry and Biology of Sialic Acids and Related Substances*, pp. 45-47. London: Cambridge University Press.

Gueimonde, M., Delgado, S., Mayo, B., Ruas-Madiedo, P., Margolles, A., & de los Reyes-Gavilán, C.G. (2004). Viability and diversity of probiotic *Lactobacillus* and *Bifidobacterium* populations included in commercial fermented milks. *Food Research International* 37 (9), 839-850.

Hassinen, J.B., Durbin, G.T., Tomarelli, R.M., & Bernhart, F.W. (1951). The minimal nutritional requirements of *Lactobacillus bifidus. Journal of Bacteriology* 62 (6), 771-777.

He, T., Priebe, M.G., Harmsen, H.J.M., Stellaard, F., Sun, X., Welling, G.W., & Vonk, R.J. (2006). Colonic fermentation may play a role in lactose intolerance in humans. *Journal of Nutrition* 136 (1), 58-63.

He, T., Priebe, M.G., Zhong, Y., Huang, C., Harmsen, H.J.M., Raangs, G.C., Antoine, J.-M., Welling, G.W., & Vonk, R.J. (2007). Effects of yogurt and bifidobacteria supplementation on the colonic microbiota in lactose-intolerant subjects. *Journal of Applied Microbiology* 104 (2), 595-604.

Heck, J.M.L., van Valenberg, H.J.F., Dijkstra, J., & van Hooijdonk, A.C.M. (2009). Seasonal variation in the Dutch bovine raw milk composition. *Journal of Diary Science* 92 (10), 4745-4755.

Henriksson, A., & Conway, P.L. (2001). Isolation of human faecal bifidobacteria which reduce signs of *Salmonella* infection when orogastrically dosed to mice. *Journal of Applied Microbiology* 90 (2), 223-228.

Hernandez-Hernandez, O., Lebron-Aguilar, R., Quintanilla-Lopez, J.E., Sanz, M.L., & Moreno, F.J. (2010). Development of a new method using HILIC-tandem mass spectrometry for the characterization of *O*-sialoglycopeptides from proteolytically digested caseinomacropeptide. *Proteomics* 10 (20), 3699-3711.

Hertzler, S.R., & Savaiano, D.A. (1996). Colonic adaptation to daily lactose feeding in lactose maldigesters reduces lactose intolerance. *American Journal of Clinical Nutrition* 64 (2), 232-236.

Holland, J.W., Deeth, H.C., & Alewood, P.F. (2005). Analysis of *O*-glycosylation site occupancy in bovine κ-casein glycoforms separated by two-dimensional gel electrophoresis. *Proteomics* 5 (4), 990-1002.

Holst, H.H., & Chatterton, D.E.W. (2002). *A process for preparing a κ-caseino glycomacropeptide or a derivative thereof*. US Patent 6,462,181.

Hou, J.-W., Yu, R.-C., & Chou, C.-C. (2000). Changes in some components of soymilk during fermentation with bifidobacteria. *Food Research International* 33 (5), 393-397.

Hua, S., Nwosu, C.C., Strum, J.S., Seipert, R.R., An, H.J., Zivkovic, A.M., German, J.B., & Lebrilla, C.B. (2012). Site-specific protein glycosylation analysis with glycan isomer differentiation. *Analytical and Bioanalytical Chemistry* 403 (5), 1291-1302.

Ibrahim, S.A., & Bezkorovainy, A. (1993). Survival of bifidobacteria in the presence of bile salt. *Journal of the Science of Food and Agriculture* 62 (4), 351-354.

Idota, T., Kawakami, H., & Nakajima, I. (1994). Growth-promoting effects of *N*-acetylneuraminic acid-containing substances on bifidobacteria. *Bioscience, Biotechnology, and Biochemistry* 58 (9), 1720-1722.

Inoue, S., Lin, S.-L., Chang, T., Wu, S.-H., Yao, C.-W., Chu, T.-Y., Troy, F.A., & Inoue, Y. (1998). Identification of free deaminated sialic acid (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid) in human red blood cells and its elevated expression in fetal cord red blood cells and ovarian cancer cells. *Journal of Biological Chemistry* 273 (42), 27199-27204.

Janer, C., Peláez, C., & Requena, T. (2004). Caseinomacropeptide and whey protein concentrate enhance *Bifidobacterium lactis* growth in milk. *Food Chemistry* 86 (2), 263-267.

Jeon S.G., Kayama H., Ueda Y., Takahashi T., Asahara T., Tsuji H., Noriko, M.T., Kiyono, H., Ma, J.S., Kusu, T., Okumura, R., Hara, H., Yoshida, H., Yamamoto, M., Nomoto, K., & Takeda, K. (2012) Probiotic *Bifidobacterium breve* induces IL-10-producing Tr1 cells in the colon. *PLoS Pathogens* 8(5), e1002714.

Jers, C., Michalak, M., Larsen, D.M., Kepp, K.P., Li, H., Guo, Y., Kirpekar, F., Meyer, A.S., & Mikkelsen, J.D. (2014). Rational design of a new *Trypanosoma rangeli* trans-sialidase for efficient sialylation of glycans. *PLoS ONE* 9 (1), e83902.

Jiskoot, W., & Crommelin, D. (2005). *Methods for Structural Analysis of Protein Pharmaceuticals*, pp. 314-315. New York: Springer Science & Business Media.

Jollès, J., Schoentgen, F., Alais, C., & Jollès, P. (1972). Studies on the primary structure of cow kappa-casein: The primary sequence of cow para-kappa-casein. *Chimia (Aarau)* 26 (6), 645-646.

Karav, S., Le Parc, A., Leite Nobrega de Moura Bell, J.M., Frese, S.A., Kirmiz, N., Block, D.E., Barile, D., & Mills, D.A. (2016). Oligosaccharides released from milk glycoproteins are selective growth substrates for infant-associated bifidobacteria. *Applied and Environmental Microbiology* 82 (12), 3622-3630.

Kawasaki, Y., Dosako, S., Shimatani, M., & Idota, T. (1994). *Process for producing k-casein glycomacropeptides*. US Patent 5,280,107.

Kim, G.B., Yi, S.H., & Lee, B.H. (2004). Purification and characterization of three different types of bile salt hydrolases from *Bifidobacterium* strains. *Journal of Dairy Science* 87 (2), 258-266.

Kitaoka, M., Tian, J., & Nishimoto, M. (2005). Novel putative galactose operon involving lacto-*N*-biose phosphorylase in *Bifidobacterium longum*. *Applied and Environmental Microbiology* 71 (6), 3158-3162.

Kiyohara, M., Tanigawa, K., Chaiwangsri, T., Katayama, T., Ashida, H., & Yamamoto, K. (2011). An *exo*-α-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. *Glycobiology* 21 (4), 437-447.

Kiyohara, M., Nakatomi, T., Kurihara, S., Fushinobu, S., Suzuki, H., Tanaka, T., Shoda, S.-I., Kitaoka, M., Katayama, T., Yamamoto, K., & Ashida, H. (2012). α-*N*-acetylgalactosaminidase from infant-associated bifidobacteria belonging to novel glycoside hydrolase family 129 is implicated in alternative mucin degradation pathway. *Journal of Biological Chemistry* 287 (1), 693-700.

Koutsioulis, D., Landry, D., & Guthrie, E.P. (2008). Novel endo-α-*N*-acetylgalactosaminidases with broader substrate specificity. *Glycobiology* 18 (10), 799-805.

Kreuβ, M., Strixner, T., & Kulozik, U. (2009). The effect of glycosylation on the interfacial properties of bovine caseinomacropeptide. *Food Hydrocolloids* 23 (7), 1818-1826.

Lee, J.-H., & O'Sullivan, D.J. (2010). Genomic insights into bifidobacteria. *Microbiology and Molecular Biology Reviews* 74 (3), 378-416.

Liang, Y., Yan, C., Guo, Q., Xu, J., & Hu, H. (2016). Spectrophotometric determination of ammonia nitrogen in water by flow injection analysis based on NH_3-*o*-phthaldehyde-Na_2SO_3 reaction. *Analytical Chemistry Research* 10, 1-8.

Locascio, R.G., Ninonuevo, M.R., Freeman, S.L., Sela, D.A., Grimm, R., Lebrilla, C.B., Mills, D.A., & German, J.B. (2007). Glycoprofiling of bifidobacterial consumption of human milk oligosaccharides demonstrates strain specific, preferential consumption of small chain glycans secreted in early human lactation. *Journal of Agricultural and Food Chemistry* 55 (22), 8914-8919.

López, P., González-Rodríguez, I., Sánchez, B., Ruas-Madiedo, P., Suárez, A., Margolles, A., & Gueimonde, M. (2012). Interaction of *Bifidobacterium bifidum* LMG13195 with HT29 cells influences regulatory-T-cell-associated chemokine receptor expression. *Applied and Environmental Microbiology* 78 (8), 2850-2857.

Lourens-Hattingh, A., & Viljoen, B.C. (2001). Yogurt as probiotic carrier food. *International Dairy Journal* 11 (1-2), 1-17.

Macwan, S.R., Dabhi, B.K., Parmar, S.C., & Aparnathi, K.D. (2016). Whey and its utilization. *International Journal of Current Microbiology and Applied Sciences* 5 (8), 134-155.

Manso, M.A., & López-Fandiño, R. (2004). K-casein macropeptides from cheese whey: Physicochemical, biological, nutritional, and technological features for possible uses. *Food Reviews International* 20 (4), 329-355.

Marotti, I., Bonetti, A., Biavati, B., Catizone, P., & Dinelli, G. (2007). Biotransformation of common bean (*Phaseolus vulgaris* L.) flavonoid glycosides by *Bifidobacterium* species from human intestinal origin. *Journal of Agricultural and Food Chemistry* 55 (10), 3913-3919.

Martínez, F.A.C., Balciunas, E.M., Converti, A., Cotter, P.D., & de Souza Oliveira, R.P. (2013). Bacteriocin production by *Bifidobacterium* spp. A review. *Biotechnology Advances* 31 (4), 482-488.

Matsuki, T., Watanabe, K., Tanaka, R., Fukuda, M., & Oyaizu, H. (1999). Distribution of bifidobacterial species in human intestinal microflora examined with 16S rRNA-gene-targeted species-specific primers. *Applied and Environmental Microbiology* 65 (10), 4506-4512.

Mattarelli, P., Bonaparte, C., Pot, B., & Biavati, B. (2008). Proposal to reclassify the three biotypes of *Bifidobacterium longum* as three subspecies: *Bifidobacterium longum* subsp. *longum* subsp. nov., *Bifidobacterium longum* subsp. *infantis* comb. nov and *Bifidobacterium longum* subsp *suis* comb. nov.. *International Journal of Systematic and Evolutionary Microbiology* 58 (Pt 4), 767-772.

Matteuzzi, D., Crociani, F., Emaldi, O., Selli, A., & Viviani, R. (1976). Isoleucine production in bifidobacteria. *European Journal of Applied Microbiology* 2 (3), 185-194.

Miglioranza, S.B., Miglioranza, L.H., Henrique, F.C., Pitelli, P.T., Lozovoy, M.A., Simão, A.N., & Dichi, I. (2015). The role of probiotics on each component of the metabolic syndrome and other cardiovascular risks. *Expert Opinion on Therapeutic Targets* 19 (8), 1127-1138.

Mikkelsen, T.L., Frøkiær, H., Topp, C., Bonomi, F., Iametti, S., Picariello, G., Ferranti, P., & Barkholt, V. (2005). Caseinomacropeptide self-association is dependent on whether the peptide is free or restricted in κ-casein. *Journal of Dairy Science* 88 (12), 4228-4238.

Milani, C., Lugli, G.A., Duranti, S., Turroni, F., Mancabelli, L., Ferrario, C., Mangifesta, M., Hevia, A., Viappiani, A., Scholz, M., Arioli, S., Sanchez, B., Lane, J., Ward, D.V., Hickey, R., Mora, D., Segata, N., Margolles, A., van Sinderen, D., & Ventura, M. (2015). Bifidobacteria exhibit social behavior through carbohydrate resource sharing in the gut. *Scientific Reports* 5, 15782.

Modler, H.W. (1993). Bifidogenic factors – sources, metabolism and applications. *International Dairy Journal* 4 (5), 383-407.

Mollé, D., & Léonil, J. (2005). Quantitative determination of bovine κ-casein macropeptide in dairy products by liquid chromatography/electrospray coupled to mass spectrometry (LC-ESI/MS) and liquid chromatography/electrospray coupled to tamdem mass spectrometry (LC-ESI/MS/MS). *International Dairy Journal* 15 (5), 419-428.

Muniandy, S., Qvist, R., Zaini, A., Chinna, K., & Ismail, I.S. (2005). A re-evaluation of plasma sialic acid determination using the periodate resorcinol method versus the enzymatic method. *The Southeast Asian Journal of Tropical Medicine and Public Health* 36 (4), 1011-1013.

Nakamura, I., Ohmura, Y., Nagami, Y., Kamihara, T., & Fukui, S. (1982). Thiamin accumulation and growth inhibition in yeasts. *Journal of General Microbiology* 128 (11), 2601-2609.

Nakano, T., & Ozimek, L. (2000). Purification of glycomacropeptide from caseinate hydrolysate by gel chromatography and treatment with acidic solution. *Journal of Food Science* 65 (4), 588-590.

NCBI (n.d.). Taxonomy browser. Retrieved from https://www.ncbi.nlm.nih.gov/Taxonomy/Browser on May 30, 2017.

Niers, L.E.M., Timmerman, H.M., Rijkers, G.T., van Bleek, G.M., van Uden, N.O.P., Knol, E.F., Kapsenberg, M.L., Kimpen, J.L.L., & Hoekstra, M.O. (2005). Identification of strong interleukin-10 inducing lactic acid bacteria which down-regulate T helper type 2 cytokines. *Clinical and Experimental Allergy* 35 (11), 1481-1489.

Noda, H., Akasaka, N., & Ohsuqi, M. (1994). Biotin production by bifidobacteria. *Journal of Nutritional Science and Vitaminology* 40 (2), 181-188.

Nordic Council of Ministers (2012). *Nordic Nutrition Recommendations 2012*, pp. 591-600. Copenhagen: Narayana Press.

O'Kennedy, R. (1988). The determination of sialic acid – an experiment that demonstrates many important aspects of spectrophotometric assays. *Biochemical Education* 16 (1), 47-48.

O'Riordan, N., Kane, M., Joshi, L., & Hickey, R.M. (2014). Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* 24 (3), 220-236.

Odamaki, T., Horigome, A., Sugahara, H., Hashikura, N., Minami, J., Xiao, J.-Z., & Abe, F. (2015). Comparative genomics revealed genetic diversity and species/strain-level differences in carbohydrate metabolism of three probiotic bifidobacterial species. *International Journal of Genomics* 2015, 567809.

Patt, L.M., & Grimes, W.J. (1974). Cell surface glycolipid and glycoprotein glycosyltransferases of normal and transformed cells. *Journal of Biological Chemistry* 249 (3), 4157-4165.

Petschow, B.W., & Talbott, R.D. (1990). Growth promotion of *Bifidobacterium* species by whey and casein fractions from human and bovine milk. *Journal of Clinical Microbiology* 28 (2), 287-292.

Pisano, A., Packer, N.H., Redmond, J.W., Williams, K.L., & Gooley, A.A. (1994). Characterization of *O*-linked glycosylation motifs in the glycopeptide domain of bovine kappa-casein. *Glycobiology* 4 (6), 837-844.

Poch, M., & Bezkorovainy, A. (1991). Bovine milk κ-casein trypsin digest is a growth enhancer for the genus *Bifidobacterium. Journal of Agricultural and Food Chemistry* 39 (1), 73-77.

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Matteuzzi, D., & Rossi, M. (2007). Folate production by bifidobacteria as a potential probiotic property. *Applied and Environmental Microbiology* 73 (1), 179-185.

Raimondi, S., Anighoro, A., Quartieri, A., Amaretti, A., Tomás-Barberán, F.A., Rastelli, G., & Rossi, M. (2015). Role of bifidobacteria in the hydrolysis of chlorogenic acid. *Microbiology Open* 4 (1), 41-52.

Robitaille, G. (2013). Growth-promoting effects of caseinomacropeptide from cow and goat milk on probiotics. *Journal of Dairy Research* 80 (1), 58-63.

Robitaille, G., Ng-Kwai-Hang, K.F., & Monardes, H.G. (1991). Variation in the *N*-acetyl neuraminic acid content of bovine κ-casein. *Journal of Dairy Research* 58 (1), 107-114.

Rodríguez, J.M., Murphy, K., Stanton, C., Ross, R.P., Kober, O.I., Juge, N., Avershina, E., Rudi, K., Narbad, A., Jenmalm, M.C., Marchesi, J.R., & Collado, M.C. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microbial Ecology in Health and Disease* 26, 26050.

Said, H.M. (2011). Intestinal absorption of water-soluble vitamins in health and disease. *The Biochemical Journal* 437 (3), 357-372.

Saito, T., & Itoh, T. (1992). Variations and distributions of O-glycosidically linked sugar chains in bovine κ-casein. *Journal of Dairy Science* 75 (7), 1768-1774.

Saito, T., Yamaji, A., & Itoh, T. (1991). A new isolation method of caseinoglycopeptide from sweet cheese whey. *Journal of Dairy Science* 74 (9), 2831-2837.

Saleh, F.A., & El-Sayed, E.M. (2005). Isolation and characterization of bacteriocins produced by *Bifidobacterium lactis* Bb-12 and *Bifidobacterium longum* Bb-46. In: Abd El-Salam, M.H., Kholif, A.M., Abdou, S.M., & El-Shibiny, S. (eds.). 9th Egyptian Conference for Dairy Science and Technology; 9.-11.10.2004; Egypt. Cairo: Research Papers. p 323-337.

Schauer, R. (2012). *Sialic Acids: Chemistry, Metabolism, and Function*, pp. 78-92. Wien: Springer Science & Business Media.

Schell, M.A., Karmirantzou, M., Snel, B., Vilanova, D., Berger, B., Pessi, G., Zwahlen, M.-C., Desiere, F., Bork, P., Delley, M., Pridmore, R.D., & Arigoni, F. (2002). The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. *Proceedings of the National Academy of Sciences of the United States of America* 99 (22), 14422-14427.

Schiffrin, E.J., Brassart, D., Servin, A.L., Rochat, F., & Donnet-Hughes, A. (1997). Immune modulation of blood leukocytes in humans by lactic acid bacteria: Criteria for strain selection. *American Journal of Clinical Nutrition* 66 (2), 515S-520S.

Sela, D.A., Chapman, J., Adeuya, A., Kim, J.H., Chen, F., Whitehead, T.R., Lapidus, A., Rokhsar, D.S., Lebrilla, C.B., German, J.B., Price, N.P., Richardson, P.M., & Mills, D.A. (2008). The genome sequence of *Bifidobacterium longum* subsp. *infantis* reveals adaptations for milk utilization within the infant microbiome. *Proceedings of the National Academy of Sciences of the United States of America* 105 (48), 18964-18969.

Shah, N. P. (2002). Bacteria, Beneficial – *Bifidobacterium* spp.: Morphology and physiology. *In* "Encyclopedia of Diary Sciences" (J.W. Fuquay, P.F. Fox, and P.L.H. McSweeney, eds.), Ed. II, pp. 381-394. New York: Academic Press.

Shu, Q., Qu, F., & Gill H.S. (2001). Probiotic treatment using *Bifidobacterium lactis* HN019 reduces weanling diarrhea associated with rotavirus and *Escherichia coli* infection in a piglet model. *Journal of Pediatric Gastroenterology and Nutrition* 33 (2), 171-177.

Silva-Hernandez, E.R., Nakano, T., & Ozimek, L. (2002). Isolation and analysis of kappa-casein glycomacropeptide from goat sweet whey. *Journal of Agricultural and Food Chemistry* 50 (7), 2034-2038.

Suzuki, R., Wada, J., Katayama, T., Fushinobu, S., Wakagi, T., Shoun, H., Sugimoto, H., Tanaka, A., Kumagai, H., Ashida, H., Kitaoka, M., & Yamamoto, K. (2008). Structural and thermodynamic analyses of solute-binding protein from *Bifidobacterium longum* specific for core 1 disaccharide and lacto-*N*-biose I. *Journal of Biological Chemistry* 283 (19), 13165-13173.

Takahashi, N., Kitazawa, H., Iwabuchi, N., Xiao, J.Z., Miyaji, K., Iwatsuki, K., & Saito, T. (2006). Immunostimulatory oligodeoxynucleotide from *Bifidobacterium longum* suppresses Th2 immune responses in a murine model. *Clinical and Experimental Immunology* 145 (1), 130-138.

Tang, X., Tian, Q., Cheng, X., Li, N., & Mao, X.-Y. (2013). Bifidobacterial growth-promoting effect of yak milk κ-casein hydrolysates produced with different proteases. *International Journal of Food Science and Technology* 48 (8), 1682-1687.

Thomä-Worringer, C., Sørensenb, J., & López-Fandiño, R. (2006). Health effects and technological features of caseinomacropeptide. *International Dairy Journal* 16 (11), 1324-1333.

Touré, R., Kheadr, E., Lacroix, C., Moroni, O., & Fliss, I. (2003). Production of antibacterial substances by bifidobacterial isolates from infant stool active against *Listeria monocytogenes*. *Journal of Applied Microbiology* 95 (5), 1058-1069.

Turroni, F., Bottacini, F., Foroni, E., Mulder, I., Kim, J.-H., Zomer, A., Sánchez, B., Bidossi, A., Ferrarini, A., Giubellini, V., Delledonne, M., Henrissat, B., Coutinho, P., Oggioni, M., Fitzgerald, G.F., Mills, D., Margolles, A., Kelly, D., van Sinderen, D., & Ventura, M. (2010). Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proceedings of the National Academy of Sciences of the United States of America* 107 (45), 19514-19519.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T.R., & Zink, R. (2003). Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. *Applied and Environmental Microbiology* 69 (11), 6908-6922.

Vernazza, C.L., Gibson, G.R., & Rastall, R.A. (2005). Carbohydrate preference, acid tolerance and bile tolerance in five strains of *Bifidobacterium. Journal of Applied Microbiology* 100 (4), 846-853.

Vreeman, H.J., Visser, S., Slangen, C.J., & Van Riel, J.A.M. (1986). Characterization of bovine kappa-casein fractions and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochemical Journal* 240 (1), 87-97.

Wang, Y.-C., Yu, R.-C., & Chou, C.-C. (2006). Antioxidative activities of soymilk fermented with lactic acid bacteria and bifidobacteria. *Food Microbiology* 23 (2), 128-135.

Ward, R.E., Niñonuevo, M., Mills, D.A., Lebrilla, C.B., & German, J.B. (2007). In vitro fermentability of human milk oligosaccharides by several strains of bifidobacteria. *Molecular Nutrition and Food Research* 51 (11), 1398-1405.

Warren, L., & Felsenfeld, H. (1962). The biosynthesis of sialic acids. *Journal of Biological Chemistry* 237 (5), 1421-1431.

Webb, M. (1951). The influence of magnesium on cell division. *Journal of General Microbiology* 5 (3), 480-484.

West, D.B., Delany, J.P., Camet, P.M., Blohm, F., Truett, A.A., & Scimeca, J. (1998). Effects of conjugated linoleic acid on body fat and energy metabolism in the mouse. *American Journal of Physiology* 275 (3), R667-R672.

Whitman, W., Goodfellow, M., Kämpfer, P., Busse, H.-J., Trujillo, M., Ludwig, W., & Suzuki, K. (2012). *Bergey's Manual of Systematic Bacteriology: Volume 5: The Actinobacteria*, pp. 177-198. New York: Springer Science & Business Media.

Xiao, J.Z., Kondo, S., Takahashi, N., Miyaji, K., Oshida, K., Hiramatsu, A., Iwatsuki, K., Kokubo, S., & Hosono, A. (2003). Effects of milk products fermented by *Bifidobacterium longum* on blood lipids in rats and healthy adult male volunteers. *Journal of Dairy Science* 86 (7), 2452-2461.

Yamauchi, K., Azuma, N., Kobayashi, H., & Kaminogawa, S. (1981). Isolation and properties of human κ-casein. *Journal of Biochemistry* 90 (4), 1005-1012.

Yao, K., & Ubuka, T. (1987). Determination of sialic acids by acidic ninhydrin reaction. *Acta Medica Okayama* 41 (6), 237-241.

Young, W. Egert, M., Bassett, S.A., & Bibiloni, R. (2015). Detection of sialic acid-utilising bacteria in a caecal community batch culture using RNA-based stable isotope probing. *Nutrients* 7 (4), 2109-2124.

Yvon, M., Beucher, S., Guilloteau, P., Le Huerou-Luron, I., & Corring, T. (1994). Effects of caseinomacropeptide (CMP) on digestion regulation. *Reproduction Nutrition Development* 34 (6), 527-537.

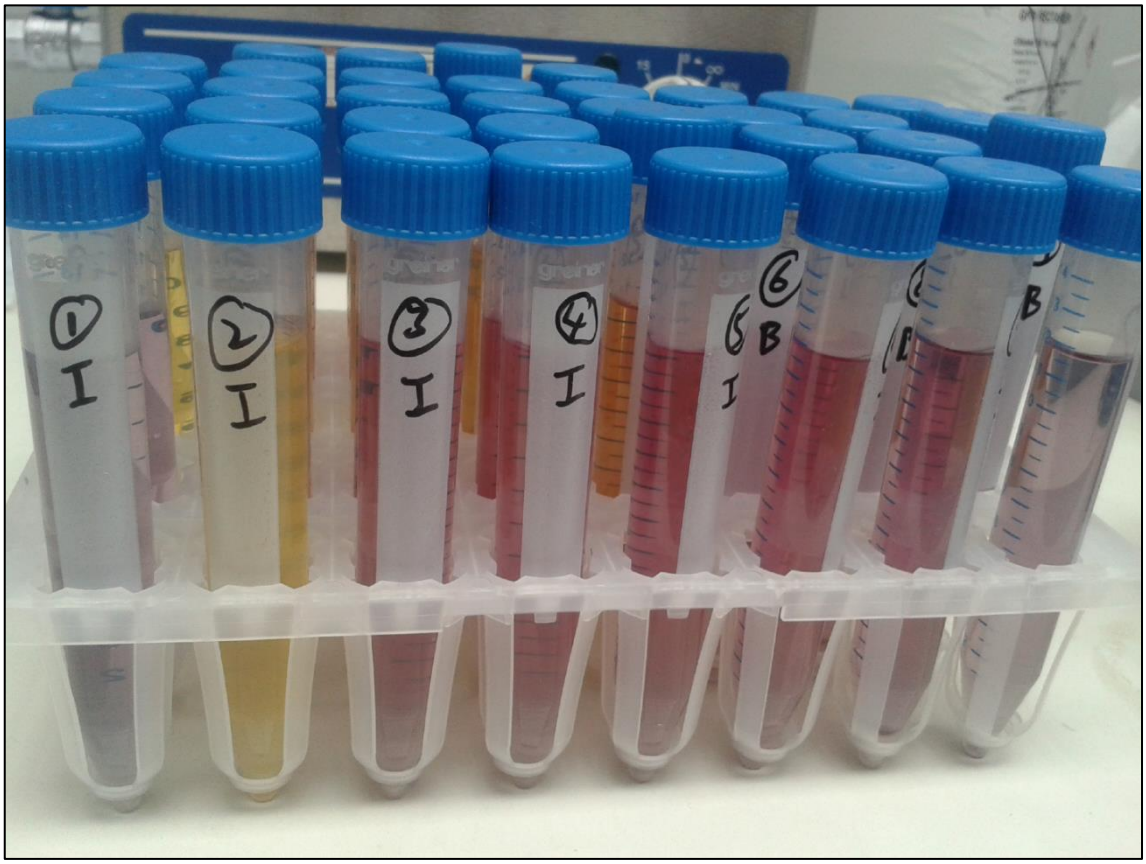
Zevaco, C., & Ribadeau-Dumas, B. (1984). A study on the carbohydrate binding sites of bovine kappa-casein using high performance liquid chromatography. *Milchwissenschaft* 39 (4), 206-210.

Zhang, B., Zhou, K., Zhang, J., Chen, Q., Liu, G., Shang, N., Qin, W., Li, P., & Lin, F. (2009). Accumulation and species distribution of selenium in Se-enriched bacterial cells of the *Bifidobacterium animalis* 01. *Food Chemistry* 115 (2), 727-734.

Zhang, D., Wei, C., Yao, J., Cai, X., & Wang, L. (2015). Interleukin-10 gene-carrying bifidobacteria ameliorate murine ulcerative colitis by regulating regulatory T cell/T helper 17 cell pathway. *Experimental Biology and Medicine* 240 (12), 1622-1629.

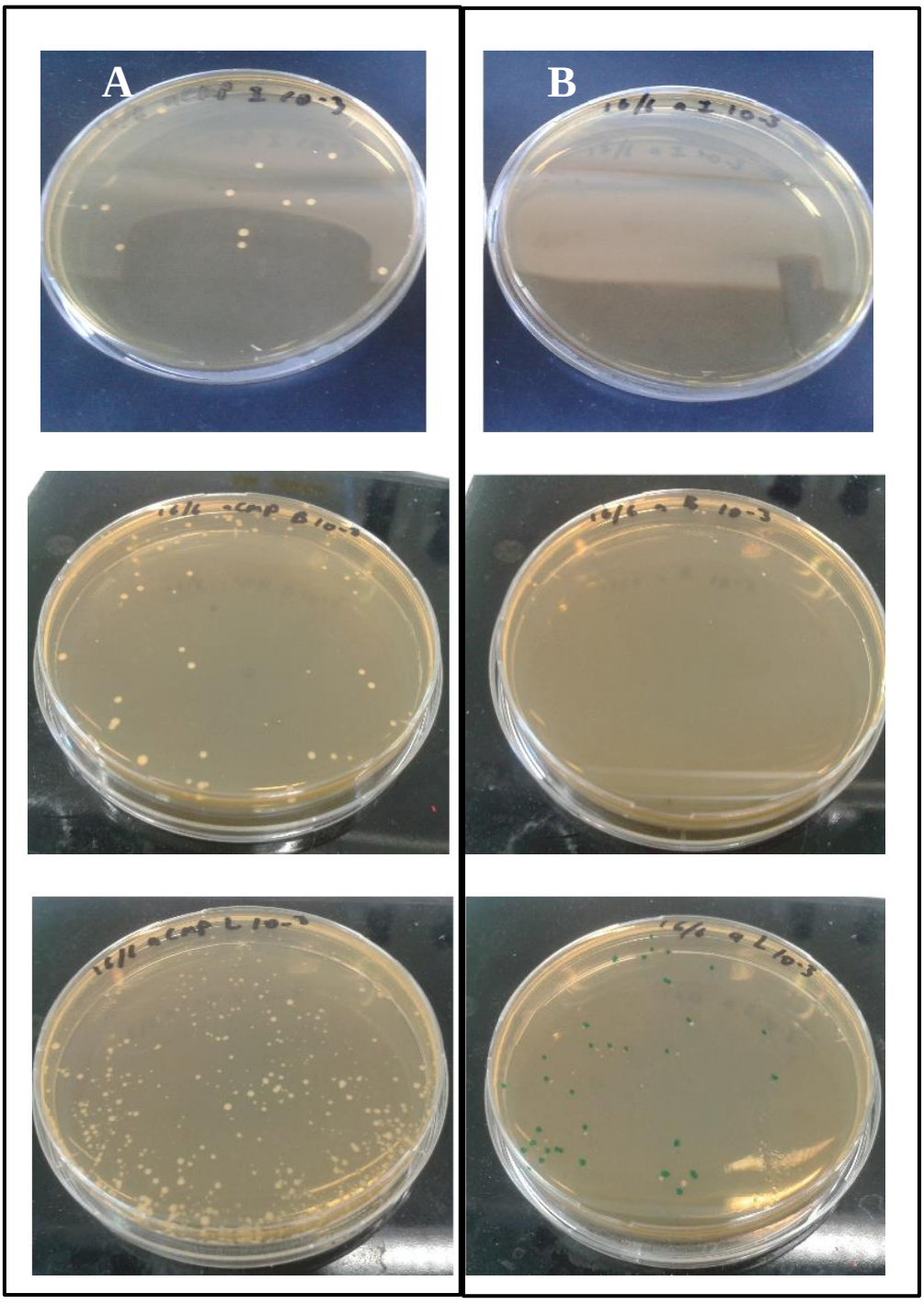
APPENDICES

Appendix 1: Preliminary experiments during bCDM development



Appendix 2: MRS agar plates of bCDM+CMP and bCDM

Plates of (A) bCDM+CMP, and (B) bCDM showing the final cell counts at 10^{-3}



Appendix 3: ANOVA test result of the final cell counts in bCDM and bCDM+CMP

Oneway

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Cell count after 38hr-incubation (B. infantis)	bCDM	2	2.00E+002	1.414E+002	1.000E+002	-1.07E+003	1.47E+003	1.E+002	3.E+002
	bCDM+CMP	2	1.05E+004	2.121E+003	1.500E+003	-8.56E+003	2.96E+004	9.E+003	1.E+004
	Total	4	5.35E+003	6.072E+003	3.036E+003	-4.31E+003	1.50E+004	1.E+002	1.E+004
Cell count after 38hr-incubation (B. bifidum)	bCDM	2	8.65E+003	4.950E+002	3.500E+002	4.20E+003	1.31E+004	8.E+003	9.E+003
	bCDM+CMP	2	9.55E+004	6.364E+003	4.500E+003	3.83E+004	1.53E+005	9.E+004	1.E+005
	Total	4	5.21E+004	5.028E+004	2.514E+004	-2.79E+004	1.32E+005	8.E+003	1.E+005
Cell count after 38hr-incubation (B. longum)	bCDM	2	3.10E+004	2.970E+004	2.100E+004	-2.36E+005	2.98E+005	1.E+004	5.E+004
	bCDM+CMP	3	1.37E+006	4.726E+005	2.728E+005	1.93E+005	2.54E+006	1.E+006	2.E+006
	Total	5	8.32E+005	8.044E+005	3.597E+005	-1.66E+005	1.83E+006	1.E+004	2.E+006

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Cell count after 38hr-incubation (B. infantis)	Between Groups	106090000.0	1	106090000.0	46.942	.021
	Within Groups	4520000.000	2	2260000.000		
	Total	110610000.0	3			
Cell count after 38hr-incubation (B. bifidum)	Between Groups	7542922500	1	7542922500	370.250	.003
	Within Groups	40745000.00	2	20372500.00		
	Total	7583667500	3			
Cell count after 38hr-incubation (B. longum)	Between Groups	2.141E+12	1	2.141E+12	14.350	.032
	Within Groups	4.475E+11	3	1.492E+11		
	Total	2.588E+12	4			

Appendix 4: ANOVA test result of the final cell counts in MRS and MRS+CMP

Oneway

Descriptives

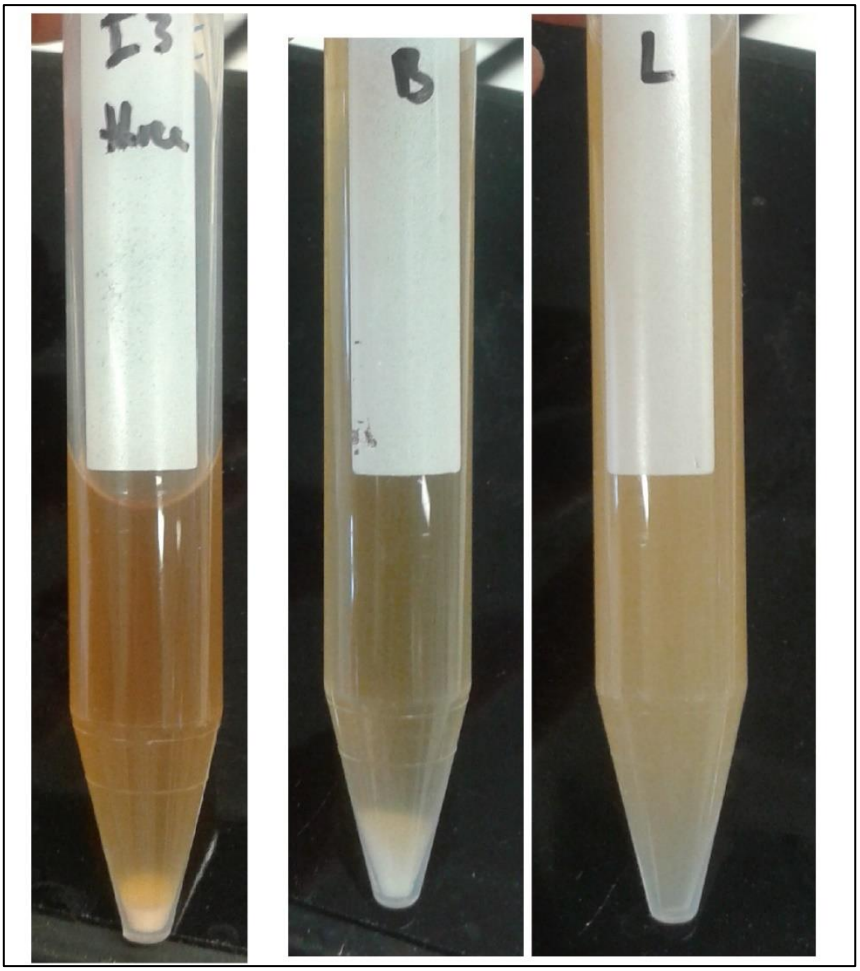
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Cell count after 22hr-incubation (B. infantis)	MRS	4	4.1000E+007	3.50428E+7	1.75214E+7	-1.4761E+7	9.6761E+007	1.20E+007	9.00E+007
	MRS+CMP	4	6.7500E+007	3.20156E+7	1.60078E+7	1.6556E+007	1.1844E+008	4.00E+007	1.00E+008
	Total	8	5.4250E+007	3.41499E+7	1.20738E+7	2.5700E+007	8.2800E+007	1.20E+007	1.00E+008
Cell count after 22hr-incubation (B. bifidum)	MRS	4	5.4325E+008	4.08667E+8	2.04334E+8	-1.0703E+8	1.1935E+009	1.03E+008	9.70E+008
	MRS+CMP	4	8.2500E+008	4.36845E+8	2.18422E+8	1.2988E+008	1.5201E+009	2.00E+008	1.15E+009
	Total	8	6.8413E+008	4.19573E+8	1.48342E+8	3.3335E+008	1.0349E+009	1.03E+008	1.15E+009
Cell count after 22hr-incubation (B. longum)	MRS	4	8.2225E+008	5.37749E+8	2.68874E+8	-3.3428E+7	1.6779E+009	9.90E+007	1.40E+009
	MRS+CMP	4	9.3500E+008	1.70000E+8	8.50000E+7	6.6449E+008	1.2055E+009	7.00E+008	1.10E+009
	Total	8	8.7863E+008	3.74098E+8	1.32264E+8	5.6587E+008	1.1914E+009	9.90E+007	1.40E+009

ANOVA

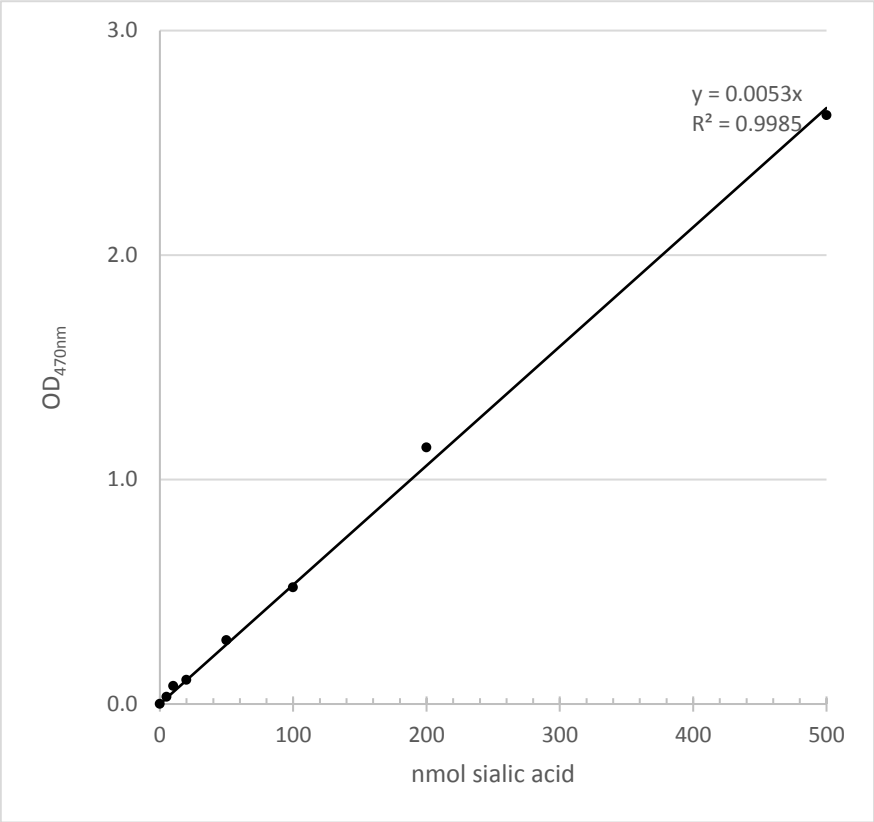
		Sum of Squares	df	Mean Square	F	Sig.
Cell count after 22hr-incubation (B. infantis)	Between Groups	1.405E+15	1	1.405E+15	1.247	.307
	Within Groups	6.759E+15	6	1.127E+15		
	Total	8.163E+15	7			
Cell count after 22hr-incubation (B. bifidum)	Between Groups	1.588E+17	1	1.588E+17	.887	.383
	Within Groups	1.074E+18	6	1.789E+17		
	Total	1.232E+18	7			
Cell count after 22hr-incubation (B. longum)	Between Groups	2.543E+16	1	2.543E+16	.160	.703
	Within Groups	9.542E+17	6	1.590E+17		
	Total	9.796E+17	7			

Appendix 5: Overnight culture tubes

Culture tubes of (from left to right) *B. infantis*, *B. bifidum*, and *B. longum*.



Appendix 6: Standard curve of sialic acid



Appendix 7: Bound sialic acid contents in bCDM and bCDM+CMP and the ANOVA test result

Specimens	Bound sialic acid contents (g/L)			Differences of bound sialic acid contents with the control
	Tube 1	Tube 2	Mean ± Standard deviation	g/L (in percentage)
			bCDM	
Control	0.013	0.007	0.010 ± 0.005	Small differences may due to random errors
B. infantis	0.006	0.008	0.007 ± 0.001	
B. bifidum	0.014	0.008	0.011 ± 0.004	
B. longum	0.004	0.005	0.005 ± 0.001	
			bCDM+CMP	
Control	1.219	1.304	1.261 ± 0.060	
B. infantis	0.977	1.355	1.166 ± 0.267	-0.095 (-7.6%)
B. bifidum	1.157	1.283	1.220 ± 0.090	-0.041 (-3.3%)
B. longum	1.307	1.244	1.244 ± 0.089	-0.017 (-1.4%)

Oneway

Descriptives

bound sialic acid in bCDM+CMP

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	2	1.26150	.060104	.042500	.72149	1.80151	1.219	1.304
B. infantis	2	1.16600	.267286	.189000	-1.23547	3.56747	.977	1.355
B. bifidum	2	1.22000	.089095	.063000	.41951	2.02049	1.157	1.283
B. longum	2	1.24400	.089095	.063000	.44351	2.04449	1.181	1.307
Total	8	1.22288	.120293	.042530	1.12231	1.32344	.977	1.355

ANOVA

bound sialic acid in bCDM+CMP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.010	3	.003	.152	.923
Within Groups	.091	4	.023		
Total	.101	7			

Appendix 8: Bound sialic acid contents in MRS and MRS+CMP and the ANOVA test result

Specimens	Bound sialic acid contents (g/L)			Differences of bound sialic acid contents with the control
	Tube 1	Tube 2	Mean ± Standard deviation	g/L (in percentage)
			MRS	
Control	0.378	0.270	0.324 ± 0.077	
B. infantis	0.266	0.280	0.273 ± 0.010	-0.051 (-15.7%)
B. bifidum	0.316	0.261	0.288 ± 0.039	-0.036 (-11.0%)
B. longum	0.240	0.282	0.261 ± 0.030	-0.063 (-19.5%)
			MRS+CMP	
Control	0.497	0.437	0.467 ± 0.042	
B. infantis	0.414	0.403	0.409 ± 0.008	-0.059 (-12.5%)
B. bifidum	0.423	0.418	0.421 ± 0.004	-0.046 (-9.9%)
B. longum	0.425	0.421	0.423 ± 0.003	-0.044 (-9.5%)

Oneway

Descriptives

bound sialic acid in MRS+CMP

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Control	2	.46700	.042426	.030000	.08581	.84819	.437	.497
B. infantis	2	.40850	.007778	.005500	.33862	.47838	.403	.414
B. bifidum	2	.42050	.003536	.002500	.38873	.45227	.418	.423
B. longum	2	.42300	.002828	.002000	.39759	.44841	.421	.425
Total	8	.42975	.028838	.010196	.40564	.45386	.403	.497

ANOVA

bound sialic acid in MRS+CMP

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.004	3	.001	2.793	.173
Within Groups	.002	4	.000		
Total	.006	7			

Appendix 9: ANOVA test result of the difference of sialic acid contents between bCDM+CMP and MRS+CMP

Descriptives

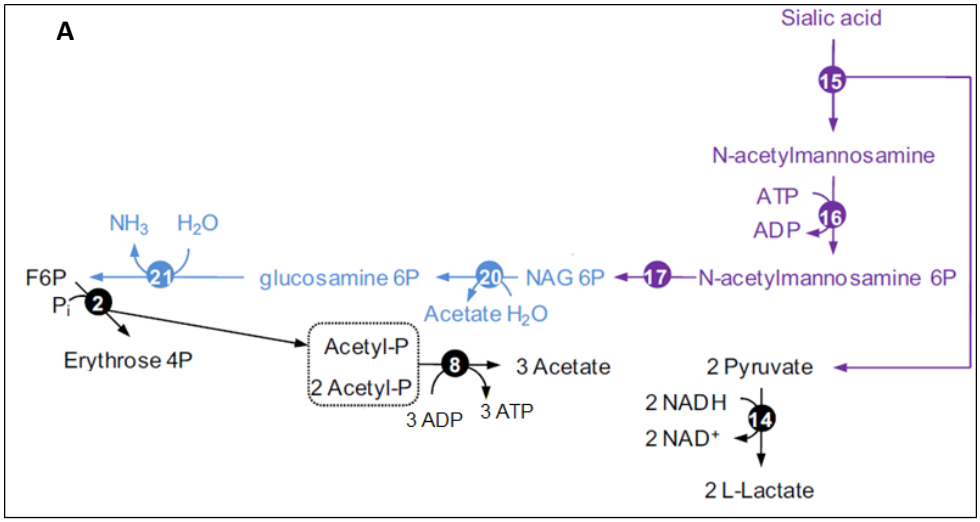
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
Diff.gSialic.L.infantis	bCDM+CMP	2	-.09550	.207182	.146500	-1.95696	1.76596	-.242	.051
	MRS+CMP	2	-.05900	.035355	.025000	-.37666	.25866	-.084	-.034
	Total	4	-.07725	.123162	.061581	-.27323	.11873	-.242	.051
Diff.gSialic.L.bifidum	bCDM+CMP	2	-.04150	.028991	.020500	-.30198	.21898	-.062	-.021
	MRS+CMP	2	-.04650	.038891	.027500	-.39592	.30292	-.074	-.019
	Total	4	-.04400	.028154	.014077	-.08880	.00080	-.074	-.019
Diff.gSialic.L.longum	bCDM+CMP	2	-.01750	.028991	.020500	-.27798	.24298	-.038	.003
	MRS+CMP	2	-.04450	.040305	.028500	-.40663	.31763	-.073	-.016
	Total	4	-.03100	.032629	.016315	-.08292	.02092	-.073	.003

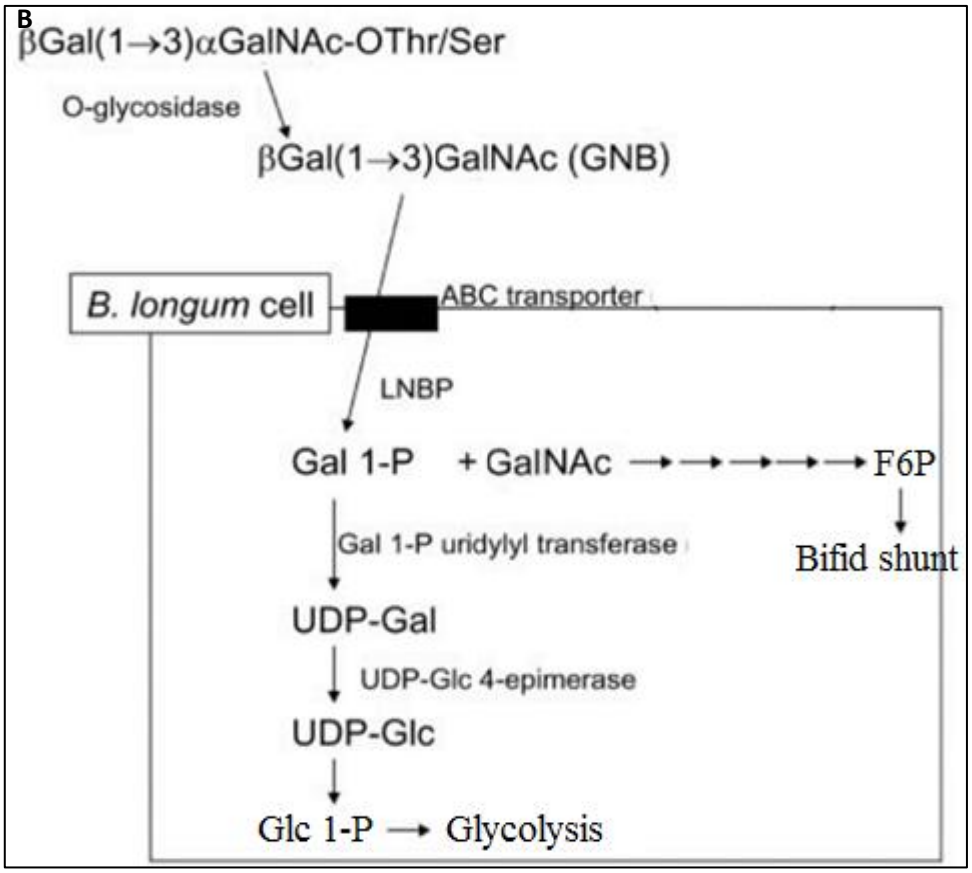
ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Diff.gSialic.L.infantis	Between Groups	.001	1	.001	.060	.829
	Within Groups	.044	2	.022		
	Total	.046	3			
Diff.gSialic.L.bifidum	Between Groups	.000	1	.000	.021	.897
	Within Groups	.002	2	.001		
	Total	.002	3			
Diff.gSialic.L.longum	Between Groups	.001	1	.001	.591	.522
	Within Groups	.002	2	.001		
	Total	.003	3			

Appendix 10: Intracellular metabolic pathways of sialic acid and GNB

Schematic metabolic pathways of (A) sialic acid in *B. infantis*, and (B) GNB in *B. longum.*





Appendix 11: Product specification of CMP

(Lacprodan CGMP-20; Arla Foods Amba, Denmark) (Page 1 of 3)

Lacprodan® CGMP-20

CASEINO GLYCO MACRO PEPTIDES

Description

Lacprodan® CGMP-20 has a high content of casein glyco macro peptides (CGMP) isolate purified specifically for application in PKU nutrition. The low Phenylalanine and the high Threonine content in combination with a pleasant taste makes Lacprodan® CGMP-20 suitable in infant formulas, clear and milky beverages and other PKU products.

Application
1. PKU
2. Clinical foods
3. Infant nutrition

Physical specifications

pH (10% solution)		6.0-7.0
Scorched particles		disc A
Solubility index	max.	1 ml
Colour of powder		white to cream
Flavour/odour		bland
Bulk Density (x625)	level	0.25 g/ cm^3

Chemical specifications

Protein (Nx6.38) as is	min.	77 %
Protein (Nx6.38) d.m.	min.	81 %
Lactose	max.	1 %
Fat	max.	1 %
Ash	max.	9 %
Moisture	max.	5.5 %
Phenylalanine (of protein)	max	0.28 %

Microbiological specifications

Total plate count	max.	10,000 CFU / g
Bacillus cereus	max.	50 CFU / g
Enterobacteriaceae	max.	10 CFU / g
Staphylococcus aureus coagulase +	absent	in 1g
Yeast/Mould	max	10 CFU/g
Salmonella	absent in	125 g

Minerals

Sodium	Na	level	1.5 %
Magnesium	Mg	level	< 0.1 %
Phosphorus	P	level	0.5 %
Chloride	Cl	level	< 0.1 %
Potassium	K	level	2.0 %
Calcium	Ca		0.1 %

Nutritional data

Calculated values for nutrition labeling per 100 g powder

Energy	1382 kJ/329kcal

Arla Foods Ingredients Group P/S
Sønderhøj 10-12, 8260 Viby J, Denmark, Tel. +45 89 38 10 00, www.arlafoodsingredients.com

Appendix 11: Product specification of CMP

(Lacprodan CGMP-20; Arla Foods Amba, Denmark) (Page 2 of 3)

Amino acids (AA)

Typical amino acid composition g. AA/100 g. protein

Alanine		6.6
Arginine		0.3
Aspartic acid (asparagine)		9.4
Cycteine (Cystin)		0.1
Glutamic acid (glutamine)		20.6
Glycine		1.2
Histidine	*	0.1
Isoleucine	*	11.3
Leucine	*	2.4
Lysine	*	6.6
Methionine	*	2.2
Phenylalanine	*	0.2
Proline		12.8
Serine		8.1
Threonine	*	18.3
Tryptophane	*	< 0.1
Tyrosine	*	< 0.03
Valine	*	9.1
Total BCAA/TAA		20.9

* Essential Aminoacids

Packaging

Paper bags with a polyethylene inner liner containing 10 kg net.

Storage

Store in closed bags under cool and dry conditions to prevent deterioration due to humidity and high temperatures.

Shelf Life

Minimum 24 months if kept under the prescribed storage conditions.

Legal references

Lacprodan® CGMP-20. The product is manufactured, packaged and labelled according to the relevant EU-regulations for food and food ingredients, and/or FAO/ WHO Codex Alimentarius, when relevant. This includes that the milk/milk constituents used as raw material origins from healthy cows. The milk used in the production is included in monitoring programmes for undesirable substances, as required by regulations or HACCP-based risk assessment. The production plants are approved by the competent authorities and included in the EU-register of approved food establishments.

For products manufactured outside EU the products comply with relevant regulations in the country where the product is produced.

Arla Foods Ingredients Group P/S
Sønderhøj 10-12, 8260 Viby J, Denmark, Tel. +45 89 38 10 00, www.arlafoodsingredients.com

Appendix 11: Product specification of CMP

(Lacprodan CGMP-20; Arla Foods Amba, Denmark) (Page 3 of 3)

GMO policy

Arla Foods objective is to avoid genetically modified ingredients in our products. The requirements we have established for our suppliers ensure that only non-GMO raw materials are used during production of our products. Therefore, our products and the raw materials used do not contain, consist of or are produced from GMO's as defined in regulation (EC) No 1829/2003, and they do not contain ingredients produced from GMO's. Therefore, our products do not need labelling according to Regulation (EC) No 1829/2003 and 1830/2003.

For the definition of GMO's, we refer to EU Directive 2001/18/EC.

Allergens

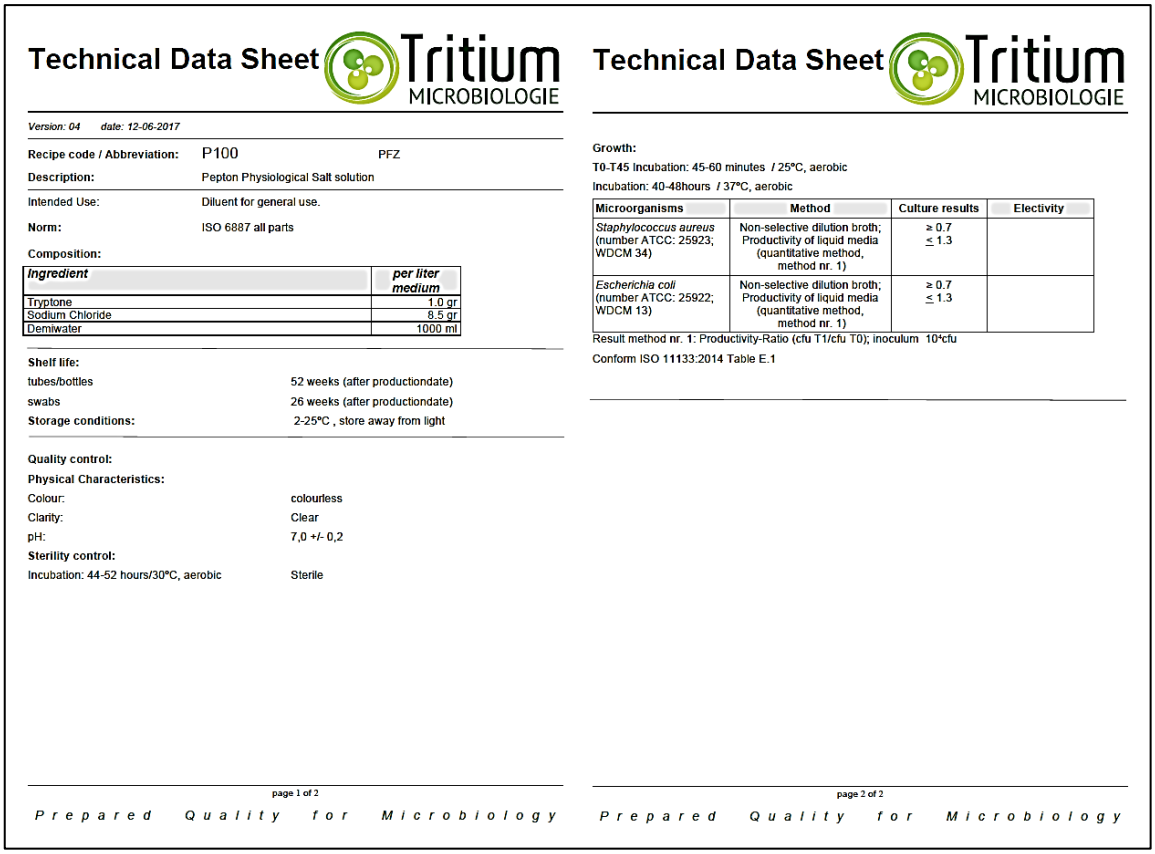
The table below indicates the presence (as added component) of the following allergens and products thereof:

YES	NO	ALLERGENS	DESCRIPTION OF COMPONENTS
	•	Cereals containing gluten and products thereof	
	•	Crustaceans and products thereof	
	•	Eggs and products Thereof	
	•	Fish and products Thereof	
	•	Peanuts and products thereof	
	•	Soya beans products thereof	
•		Milk and products thereof (including lactose	Bovine milk
		Nuts	
	•	(Tree) Nuts and products thereof	
	•	Celery and products thereof	
	•	Mustard and products thereof	
	•	Sesame seeds and products thereof	
	•	Sulphur dioxide and sulphites (>10 mg/kg)	
	•	Lupin and products thereof	
	•	Molluscs and products thereof	

Arla Foods Ingredients Group P/S
Sønderhøj 10-12, 8260 Viby J, Denmark, Tel. +45 89 38 10 00, www.arlafoodsingredients.com

Appendix 12: Product specification of PFZ

(Tritium Microbiologie, the Netherlands)



Technical Data Sheet

Tritium MICROBIOLOGIE

Version: 04 date: 12-06-2017

Recipe code / Abbreviation: P100 PFZ

Description: Pepton Physiological Salt solution

Intended Use: Diluent for general use.

Norm: ISO 6887 all parts

Composition:

Ingredient	*per liter medium*
Tryptone	1.0 gr
Sodium Chloride	8.5 gr
Demiwater	1000 ml

Shelf life:

tubes/bottles 52 weeks (after productiondate)

swabs 26 weeks (after productiondate)

Storage conditions: 2-25°C , store away from light

Quality control:

Physical Characteristics:

Colour: colourless

Clarity: Clear

pH: 7,0 +/- 0,2

Sterility control:

Incubation: 44-52 hours/30°C, aerobic Sterile

page 1 of 2

Prepared Quality for Microbiology

Technical Data Sheet

Tritium MICROBIOLOGIE

Growth:

T0-T45 Incubation: 45-60 minutes / 25°C, aerobic

Incubation: 40-48hours / 37°C, aerobic

Microorganisms	Method	Culture results	Electivity
Staphylococcus aureus (number ATCC: 25923; WDCM 34)	Non-selective dilution broth; Productivity of liquid media (quantitative method, method nr. 1)	≥ 0.7 ≤ 1.3	
Escherichia coli (number ATCC: 25922; WDCM 13)	Non-selective dilution broth; Productivity of liquid media (quantitative method, method nr. 1)	≥ 0.7 ≤ 1.3	

Result method nr. 1: Productivity-Ratio (cfu T1/cfu T0); inoculum 10^4cfu

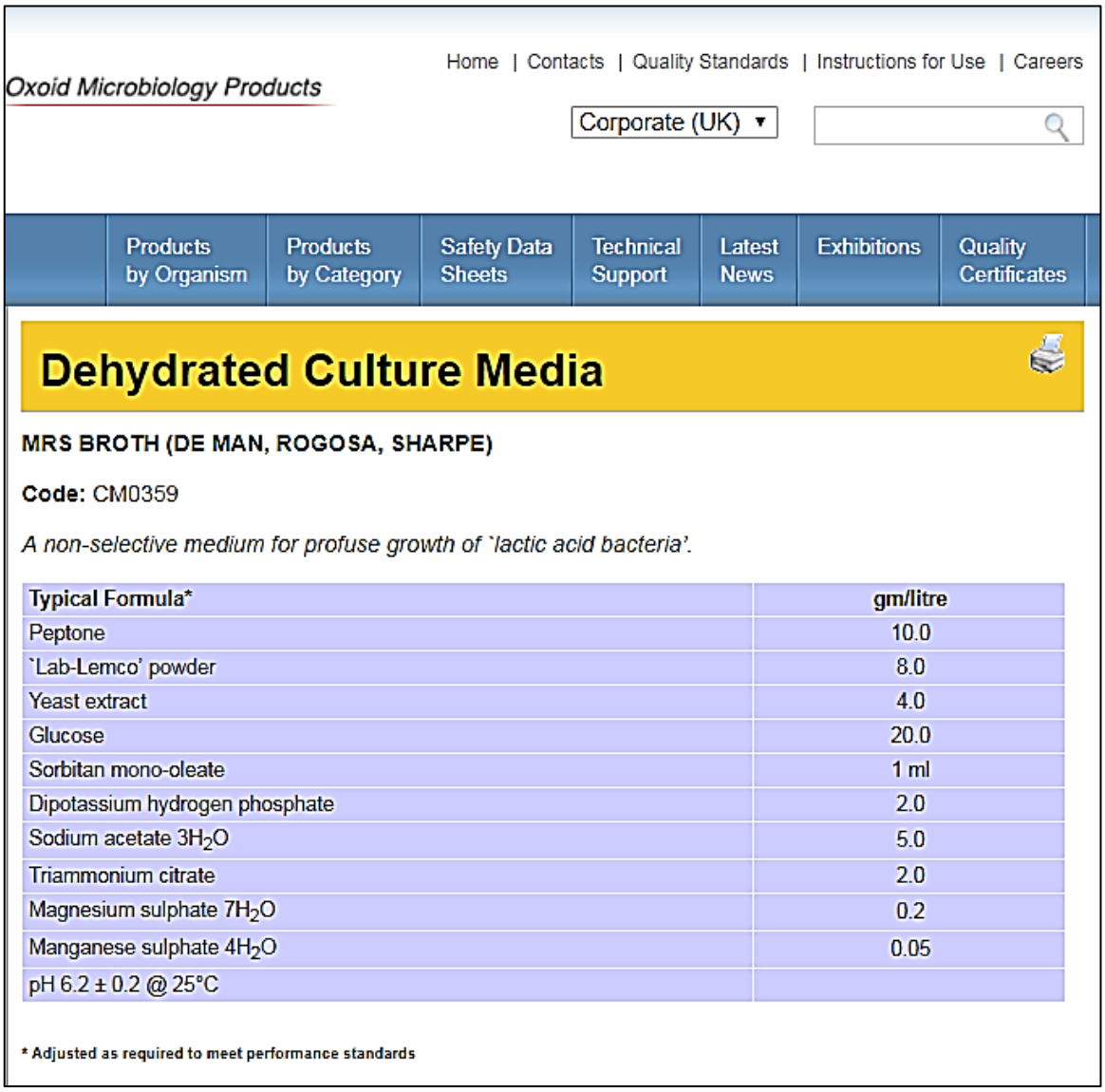
Conform ISO 11133:2014 Table E.1

page 2 of 2

Prepared Quality for Microbiology

Appendix 13: Product specification of MRS

(Oxoid, UK)



Oxoid Microbiology Products

Home | Contacts | Quality Standards | Instructions for Use | Careers

Corporate (UK)

Products by Organism | Products by Category | Safety Data Sheets | Technical Support | Latest News | Exhibitions | Quality Certificates

Dehydrated Culture Media

MRS BROTH (DE MAN, ROGOSA, SHARPE)

Code: CM0359

A non-selective medium for profuse growth of `lactic acid bacteria'.

Typical Formula*	gm/litre
Peptone	10.0
`Lab-Lemco' powder	8.0
Yeast extract	4.0
Glucose	20.0
Sorbitan mono-oleate	1 ml
Dipotassium hydrogen phosphate	2.0
Sodium acetate $3H_2O$	5.0
Triammonium citrate	2.0
Magnesium sulphate $7H_2O$	0.2
Manganese sulphate $4H_2O$	0.05
pH 6.2 ± 0.2 @ 25°C	

* Adjusted as required to meet performance standards